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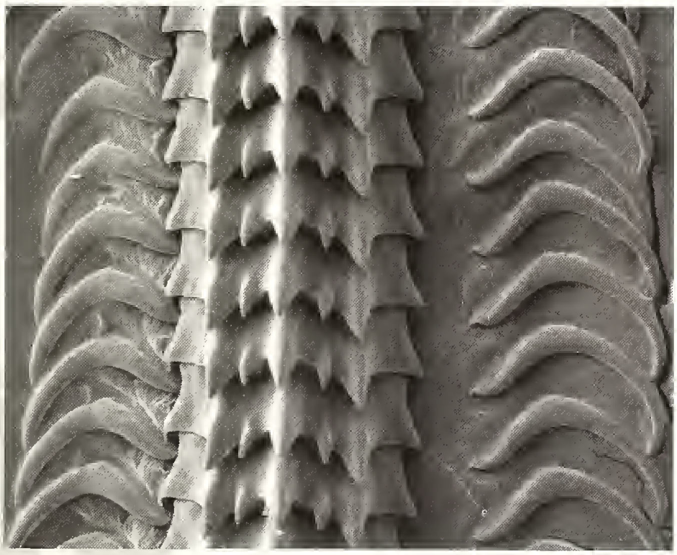

The Publications of the American Malacological Union/Society.
EUGENE V. COAN and ALAN R. KABAT 1

Taxonomic occurrences of gastropod spermatozeugmata and non-stylommatophoran spermatophores updated. **ROBERT ROBERTSON** 11

A developmental perspective on evolutionary innovation in the radula of the predatory neogastropod family Muricidae.
GREGORY S. HERBERT, DIDIER MERLE, and CARLOS S. GALLARDO 17

Phylogenetic relationships of the columbellid taxa *Cotonopsis* and *Cosmioconcha* (Neogastropoda: Buccinoidea: Columbellidae). **HELENA FORTUNATO** 33

Family Pseudolividae (Caenogastropoda, Muricoidea): A polyphyletic taxon.
LUIZ RICARDO L. SIMONE 43

Gastropod mating systems: An introduction to the symposium. **JANET L. LEONARD** 79

Reproductive behavior of the dioecious tidal snail *Cerithidea rhizophorarum* (Gastropoda: Potamididae).
MAYA TAKEUCHI, HARUMI OHTAKI, and KIYONORI TOMIYAMA 81

Causes of variation in sex ratio and modes of sex determination in the Mollusca—an overview.
YOICHI YUSA 89

continued on back cover

Cover photo: Radula of *Chicopinnatus laqueatus* (Sowerby, 1841) from Herbert *et al.*

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Box 351800
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Seattle, Washington 98195
USA

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Stony Brook, New York 11749-5245
USA

Roland C. Anderson
The Seattle Aquarium
1483 Alaskan Way
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USA

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Paul Callomon
Department of Malacology
The Academy of Natural Sciences of Philadelphia
1900 Benjamin Franklin Parkway
Philadelphia, Pennsylvania 19103-1195
USA

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MANUSCRIPT SUBMISSION, CLAIMS, AND PERMISSIONS TO REPRINT JOURNAL MATERIAL should be sent to the Editor-in-Chief:
Kenneth M. Brown, *Editor-in-Chief*
Department of Biological Sciences
Louisiana State University
Baton Rouge, Louisiana 70803
USA
Voice: 225-578-1740 • Fax: 225-578-2597
E-mail: kmbrown@lsu.edu

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AMERICAN MALACOLOGICAL BULLETIN

CONTENTS

VOLUME 23 | NUMBER 1/2

The Publications of the American Malacological Union/Society.
EUGENE V. COAN and ALAN R. KABAT 1

Taxonomic occurrences of gastropod spermatozeugmata and non-stylommatophoran spermatophores updated. **ROBERT ROBERTSON** 11

A developmental perspective on evolutionary innovation in the radula of the predatory neogastropod family Muricidae.
GREGORY S. HERBERT, DIDIER MERLE, and CARLOS S. GALLARDO 17

Phylogenetic relationships of the columbellid taxa *Cotonopsis* and *Cosmioconcha* (Neogastropoda: Buccinoidea: Columbellidae). **HELENA FORTUNATO** 33

Family Pseudolividae (Caenogastropoda, Muricoidea): A polyphyletic taxon.
LUIZ RICARDO L. SIMONE 43

Gastropod mating systems: An introduction to the symposium. **JANET L. LEONARD** 79

Reproductive behavior of the dioecious tidal snail *Cerithidea rhizophorarum* (Gastropoda: Potamididae).
MAYA TAKEUCHI, HARUMI OHTAKI, and KIYONORI TOMIYAMA 81

Causes of variation in sex ratio and modes of sex determination in the Mollusca—an overview.
YOICHI YUSA 89

Poecilogony and larval ecology in the gastropod genus *Alderia*. **PATRICK J. KRUG** 99

Food intake, growth, and reproduction as affected by day length and food availability in the pond snail *Lymnaea stagnalis*.
ANDRIES TER MAAT, COR ZONNEVELD, J. ARJAN G.M. DE VISSER, RENÉ F. JANSEN, KORA MONTAGNE-WAJER, and JORIS M. KOENE 113

Phally polymorphism and reproductive biology in *Ariolimax (Ariolimax) buttoni* (Pilsbry and Vanatta, 1896) (Stylommatophora: Arionidae).
JANET L. LEONARD, JANE A. WESTFALL, and JOHN S. PEARSE 121

A review of mating behavior in slugs of the genus *Deroceras* (Pulmonata: Agriolimacidae).
HEIKE REISE 137

Reproductive biology and mating conflict in the simultaneously hermaphroditic land snail *Arianta arbustorum*. **BRUNO BAUR** 157

A literature database on the mating behavior of stylommatophoran land snails and slugs.
ANGUS DAVISON and PETER MORDAN 173

The function of dart shooting in helicid snails. **RONALD CHASE** 183

Meeting Announcement 190

Melbourne Romaine Carriker: 25 February 1915 – 25 February 2007 An Appreciation.
CLEMENT L. COUNTS, III, ROBERT S. PREZANT, and J. EVAN WARD 191

Financial Report 195

Index to Vol. 23 197

Membership Form 201

Information for Contributors 203

Amer. Malac. Bull. **23**: 1-10

The Publications of the American Malacological Union/Society

Eugene V. Coan[1] **and Alan R. Kabat**[2]

[1] Santa Barbara Museum of Natural History, 2559 Puesta del Sol Road, Santa Barbara, California 93105, U.S.A., gene.coan@sierraclub.org

[2] Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts 02138, U.S.A., alankabat@aol.com

Abstract: This paper documents the publications of the American Malacological Society, and its predecessor, the American Malacological Union, from 1931 through 2007. Information on the dates of publication is included, based primarily on library receipt records. Several publications were erroneously dated as to year, which has resulted in new taxa described in those publications, as well as abstracts and articles, being attributed to the wrong year.

Key words: AMS, AMU, publication dates, bibliography

The American Malacological Union (AMU), now the American Malacological Society (AMS), founded in 1931, issued reports containing abstracts and papers presented at its annual meetings from its earliest years. At first these reports were published in *The Nautilus*. Shortly thereafter, reports in *The Nautilus* were supplemented by a printed report mailed to all members after the annual meeting that included a membership list. By 1941, *The Nautilus* no longer included meeting reports. The titles of the AMU's reports changed several times over the years (Bieler and Kabat 1991-present). During World War II, when no meetings were held, reports were issued to ensure communication and continuity.

In 1971, the AMU's publication became known as the *Bulletin*. In 1983, the *Bulletin* became a peer-reviewed scientific journal, the *American Malacological Bulletin*, with an increasing number of original articles. By 1985, the *American Malacological Bulletin* no longer included reports on and abstracts of papers presented at the annual meeting, which were published only in the program and abstracts volume issued to meeting participants, with the organizer of each meeting generally responsible for its publication. By this time, the membership list was no longer included in the annual report, and was instead published as part of, or in connection with, the organization's newsletter.

The *American Malacological Union Newsletter* began in 1968 and was issued irregularly. Its name changed to the *AMU News* in 1984, to *American Malacological Newsletter* in 1996, thence to *American Malacological Society Newsletter* in 1999, and finally to the *Newsletter of the American Malacological Society* in 2003. The newsletter is now circulated chiefly electronically and is available on the AMS website: http://www.malacological.org/. In 2003, the membership list moved from the newsletter to become a separate, electronically distributed publication.

The purpose of this paper is to provide the publication dates of AMU/AMS publications. In many cases, incorrect dates were printed on them, and some issues were misnumbered. As a result, abstracts, papers, and even new taxa that appear in this series are sometimes misdated in subsequent literature.

Although the AMU/AMS and its publications have been documented in three histories (Murray 1999, Teskey 1964, 1982), three indices (Anonymous 1966, Counts 1988, Teskey 1975), and one listing (Anonymous 1981), none of these provided fully accurate or complete dates for the AMU/AMS publications. The Annual Report for 1967 (Report No. 34: 84) provided the dates of publication for the reports from 1961 through 1966; we are unaware of any other similar listings.

The reports appearing in *The Nautilus* from 1931 to 1941 are easily dated using the compilation of Coan and Harasewych (1993).

The individual reports and bulletins contain various kinds of dates. The most common is the date the included membership list was prepared, herein indicated by "Membership List." These cannot be taken as publication dates, although some other sources have used them, because the report was often printed and mailed weeks or months after the membership list was prepared. These dates, however, are included here for comparison and to indicate that the publication could not have been issued any earlier.

Some reports are simply dated, without further indication as to what this date means (herein "Stated Date"). Starting with volume 18 of the *American Malacological Bulletin*, the printed date, for the first time, gives both the month and day, and is intended and assumed to be the publication date, based on receipt of mailed copies within one or two weeks. In earlier years, however, these dates were demonstrably off by a year. In some cases, the printed dates are labeled as being specifically a publication date or mailing date, essentially the same thing. In the mid-1990s, several issues did not have the year on the cover or on the table of contents, but the year was included on the spine; such data, of course, are lost when the journal issues are bound, as is the case for most libraries.

It is instructive to compare any printed dates with the dates the publication was received in libraries, most of which stamp the date of receipt on each copy upon arrival. However, this is also not entirely conclusive, because there may be delays between receipt of the mail and date stamping. Moreover, some institutions with malacologists on their staffs did not separately subscribe to the AMU/AMS publications, and those institutions instead obtained their copies through donations from these persons, often months or years after publication. It appears that for some years the reports were sent by airmail to Europe and by bulk mail within the U.S., accounting for The Natural History Museum (London) having several of the earliest receipt dates.

We have examined the sets of AMU/AMS publications in the Academy of Natural Sciences of Philadelphia (ANSP), Library of Congress (LOC), National Museum of Natural History (USNM), Harvard University Museum of Comparative Zoology (MCZ), California Academy of Sciences (CAS), University of Maryland (UMd), University of Washington Fisheries and Oceanography Library (UW), and The Natural History Museum, London (BMNH). Three colleagues provided us with information on the sets at the Field Museum of Natural History (FMNH), the American Museum of Natural History (AMNH), and the Carnegie Museum of Natural History (CMNH). When these dates are consistently later than the published dates—sometimes much later—it is clear that the stated publication dates must be incorrect. If no date is given for a particular institution for a given year, this means that the institution (1) lacks the volume in question; (2) has the volume, but there is no date stamp; or (3) has the volume, but the date stamp is several weeks, months, years, or even decades later. In several cases, one of us (EVC) noted the date of receipt of his personal copies.

We have also included the three "*Special Publications*," which were issued in the 1980s as supplements to the *American Malacological Bulletin*. However, as these were not included in the regular subscription, many libraries did not order them.

When we are certain or fairly certain about publication dates, these are indicated with a single date. In other cases, we present the evidence thus far available, which in many cases indicates that the *Bulletins* were issued the year following the meeting, or months, or even a year after the internal date.

For reference, the place and dates of the meetings through 1985 are given, along with the number of the meeting. After the publications stopped covering the meetings, this information is not provided here, but is readily available online (Coan and Kabat 1996-present). Also indicated are the meetings of the Pacific Division of the AMU from its inception in 1948 until its separation as the Western Society of Malacologists in 1968.

The International Code of Zoological Nomenclature (ICZN 1999) emphasizes that editors and publishers should keep track of dates of publication in journals that may have new taxa or other nomenclatural acts:

> "Recommendation 21C. Specification of date. An editor or publisher should state the date of publication of a work, and of each component part of a serial publication, and of any work issued in parts. In a volume made up of parts brought out separately, the day of publication of each part, and the exact pages, plates, maps, etc. that constitute it, should be specified" (p. 23).

This paper is designed to bring the AMU/AMS publications into retroactive compliance with the *Code*.

In the recent catalog of family and higher names in the Gastropoda, and elsewhere, the subfamily names Pseudomelatominae Morrison and the (unavailable) Lophiotominae and Crassispirinae Morrison are dated from 1 December 1965, the date printed on AMU *Annual Reports* for 1965 (Bouchet and Rocroi 2005: 57, 101, 144, 256), but this report was not actually published until 28 February 1966, as evidenced in the list below. Four papers by Morrison in AMU publications are misdated in the bibliography of his works by Rosewater (1984), including this paper on the Turridae.

From the list below, it is clear that publication dates have been incorrectly stated, even in recent years. It is obvious that in spite of a "publication date" of 30 December 1976, on the cover of the report for 1976, a January 1977 publication/mailing date is more likely because no one received this issue until early February 1977. Fortunately, no new taxa appear to be included. Similarly, *American Malacological Bulletin* 3(1) is dated December 1984, but no institutions received it until mid-February 1985, and a late January or early February 1985 publication date is more likely. No new taxa were included in this issue. The issue *AMB* 8(1) was dated August 1990, but it appears not be have been received anywhere before early October, so a publication date of mid- to late-September seems more probable. Again, no new taxa were included in this issue. However, *AMB* 17(1/2), indicated as having been published in December 2002, appears to have actually been issued in February 2003. This issue contains new taxa of marine and terrestrial gastropods, as well as a species of fossil rostroconch, and all of these should evidently be dated 2003 rather than 2002.

ANNUAL REPORTS AND BULLETIN

Notes

* Publication dates of *The Nautilus* from Coan and Harasewych (1993).

n/a = not applicable.

# Issue numbers printed on some of the *Bulletins*, but incorrectly numbered and not corresponding either to the number of the meeting or to the sequential number of separately issued bulletins.

The Nautilus; American Malacological Union [*Report*]

Meeting	Publication	Earliest Date of Publication	Later Receipt Dates	Comments
1 (Philadelphia, Pennsylvania, 30 April-2 May 1931)	*The Nautilus* 45(1): 1-5	13 July 1931	n/a	Publication date: Coan and Harasewych (1993)
n/a	"Members of the American Malacological Union," 5 pages	1 April 1932	n/a	Date on cover
2 (Washington, D.C., 26-28 May 1932)	*The Nautilus* 46(1): 1-3	23 July 1932	n/a	Publication date: Coan and Harasewych (1993)
	"Mrs. Imogene C. Robertson's Rambling Notes", 11 pages	1932	n/a	Undated, presumably issued in 1932
3 (Cambridge, Massachusetts, 25-27 May 1933)	*The Nautilus* 47(1): 37-44	16 June 1933	n/a	Publication date: Coan and Harasewych (1993)
4 (Stanford, California, 25-28 June 1934)	*The Nautilus* 48(2): 72	15 October 1934	n/a	Publication date: Coan and Harasewych (1993)
	Report, 12 pages	ANSP = 16 October 1934	n/a	Stated date = 1 August 1934
5 (Buffalo, New York, 27-29 June 1935)	*The Nautilus* 49(2): 62-63	8 November 1935	n/a	Publication date: Coan and Harasewych (1993)
	Report, 10 pages, 1 plate	not known; possibly late 1935 or 1936	n/a	[no data available]
6 (St. Petersburg, Florida, 21-24 April 1936)	*The Nautilus* 51(1): 33-36	14 July 1936	n/a	Publication date: Coan and Harasewych (1993)
	The American Malacological Union [Report], 14 pages	MCZ = 21 October 1936	n/a	Membership List = 1 September 1936
7 (Ann Arbor, Michigan, 3-5 August 1937)	*The Nautilus* 51(2): 68-71	22 October 1937	n/a	Publication date: Coan and Harasewych (1993)
	The American Malacological Union [Report], 16 pages	MCZ = 26 February 1938		Membership List = 1 January 1938
8 (Havana, Cuba, 1-6 August 1938)	*The Nautilus* 52(2): 66-72	28 October 1938	n/a	Publication date: Coan and Harasewych (1993)
	The American Malacological Union [Report], 20 pages, frontispiece	MCZ = 28 April 1939		Membership List = 1 January 1939

Meeting	Publication	Earliest Date of Publication	Later Receipt Dates	Comments
9 (Toronto, Ontario, Canada, 20-23 June 1939)	*The Nautilus* 53(1): 36; *The Nautilus* 53(2): 68-72	21 July 1939; 20 October 1939	n/a	Publication date: Coan and Harasewych (1993)
	The American Malacological Union [Report], 16 pages	MCZ = 2 January 1940		Membership List = November 1939
10 (Philadelphia, Pennsylvania, 17-21 June 1940)	*The Nautilus* 54(1): 35-37	23 July 1940	n/a	Publication date: Coan and Harasewych (1993)
	The American Malacological Union [Report], 18 pages	MCZ = 17 January 1941		Membership List = December 1940
11 (Rockland and Thomaston, Maine, 26-29 August 1941)	*The Nautilus* 55(2): 70-72	24 October 1941	n/a	Publication date: Coan and Harasewych (1993)
	The American Malacological Union [Report], 44 pages	Probably 1942		Membership List = January 1942
1942-1943 – No meetings				

News Bulletin and Annual Report

Meeting	Publication	Earliest Date of Publication	Later Receipt Dates	Comments
1944 – No meeting	*News Bulletin and Annual Report*, 24 pages, frontispiece	Probably 1944		Membership List = January 1944
1945 – No meeting	*News Bulletin and Annual Report*, 21 pages, frontispiece	MCZ = 26 November 1945		Membership List = October 1945
12 (Washington, D.C., 14-16 August 1946)	*News Bulletin and Annual Report*, 22 pages, frontispiece	MCZ = 22 April 1947; ANSP = 22 April 1947		Membership List = April 1947
13 (Pacific Grove, California, 18-21 June 1947)	*News Bulletin and Annual Report*, 32 pages	CMNH = 20 February 1948		Membership List = December 1947
14 (Pittsburgh, Pennsylvania, 25-27 August 1948); 1, Pacific Division (Los Angeles, California, 10-11 April 1948)	*News Bulletin and Annual Report*, 36 pages	MCZ = 9 April 1949	ANSP = 18 April 1949	Membership List = March 1949
15 (Coral Gables, Florida, 16-18 June 1949); 2, Pacific Division (Long Beach, California, 14-16 June 1949)	*News Bulletin and Annual Report*, 36 pages	MCZ = 1 February 1950	ANSP = 6 February 1950	Membership List = November 1949
16 (Chicago, Illinois, June 14-16, 1950); 3, Pacific Division (Santa Barbara, California, 7-9 April 1950)	*News Bulletin and Annual Report*, 39 pages	MCZ = 26 January 1951	ANSP = 1 February 1951	Membership List = December 1950

Meeting	Publication	Earliest Date of Publication	Later Receipt Dates	Comments
17 (Buffalo, New York, 22-24 August 1951); 4, Pacific Division (Oakland, California, 22-24 June 1951)	*News Bulletin and Annual Report*, 41 pages	28 January 1952 ["date based on annotation by Morrison on reprint in MNHN" (Bouchet and Rocroi 2005: 333)]	ANSP = 10 March 1952; MCZ = 17 March 1952	Membership List = December 1951

Annual Report

Meeting	Publication	Earliest Date of Publication	Later Receipt Dates	Comments
18 (Cambridge, Massachusetts, 20-22 August 1952); 5, Pacific Division (Los Angeles, California, 20-22 June 1952)	*Annual Report*, 44 + [i] pages	MCZ = 4 February 1953; ANSP = 4 February 1953		Membership List = 31 December 1952 [inside front cover]
19 (Lawrence, Kansas, 25-27 June 1953); 6, Pacific Division (Pacific Grove, California, 12-13 June 1953)	*Annual Report*, 51 pages	MCZ = 15 March 1954; ANSP =15 March 1954		Stated Date / Membership List = 31 December 1953
20 (Durham, New Hampshire, 16-18 August 1954); 7, Pacific Division (Los Angeles, California, 18-19 June 1954)	*Annual Report*, 44 pages	MCZ = 21 January 1955	ANSP = 26 January 1955	Membership List = 31 December 1954
21 (New York, New York, 26-29 June 1955); 8, Pacific Division (Stanford University, California, 15-16 July 1955)	*Annual Reports* 22$^{\#}$, 58 pages	ANSP = 16 April 1956	MCZ = 17 April 1956	Stated Date and Membership List = 31 December 195
22 (San Diego, California, 11-14 July 1956); 9, Pacific Division (same)	*Annual Reports* 23$^{\#}$, 52 pages	n/a	LOC = 23 February 1958 [late]	Stated Date and Membership List, 31 December 1956
23 (Yale University, New Haven, Connecticut, 16-19 July 1957); 10, Pacific Division (Santa Barbara, California, 30 May-1 June 1957)	*Annual Reports* 24$^{\#}$, 56 pages	UMd = 28 March 1958		Stated Date = 1 January 1958
24 (Ann Arbor, Michigan, 2-6 September 1958); 11, Pacific Division (Berkeley, California, 27-29 June 1958)	*Annual Reports* 25$^{\#}$, 73 pages	UMd = 11 February 1959		Stated Date = 1 January 1959
25 (Philadelphia, Pennsylvania, 30 June-3 July 1959); 12, Pacific Division (Redlands, California, 9-12 July 1959)	*Annual Reports* 26$^{\#}$, 79 pages	UMd = 26 February 1960		Stated Date = 1 January 1960; meeting misnumbered as "26" on cover and title page

Meeting	Publication	Earliest Date of Publication	Later Receipt Dates	Comments
26 (Montreal, Quebec, Canada, 9-12 August 1960); 13, Pacific Division (Pacific Grove, California, 22-25 June 1960)	*Annual Reports* 27[#], 76 pages	EVC = February 1961	BMNH = 4 October 1961; LOC = 2 November 1961	Stated Date = 1 January 1961; meeting misnumbered as "27" on cover and title page
27 (Washington, D.C., 19-23 June 1961); 14, Pacific Division (Goleta, California, 28 June-1 July 1961)	*Annual Reports* 28[#], 81 pages	12 December 1961 (Report for 1967, 34: 84)	BMNH = 8 January 1962; LOC = 10 January 1962	Stated Date = 1 December 1961; meeting misnumbered as "28" on cover and title page
28 (St. Petersburg, Florida, 31 July-3 August 1962); 15, Pacific Division (Pacific Grove, California, 27-30 June 1962)	*Annual Reports* 29[#], 68 pages	21 December 1962 (Report for 1967, 34: 84)	LOC = 23 January 1963; BMNH = 28 January 1963	Stated Date = 1 December 1962; meeting misnumbered as "29" on cover and title page
29 (Buffalo, New York, 18-21 June 1963); 16, Pacific Division (Goleta, California, 26-29 June 1963)	*Annual Reports* 30[#], 83 pages	1 January 1964 (Report for 1967, 34: 84)	LOC = 23 January 1964; BMNH = 14 February 1964; UMd = 14 February 1964	Stated Date = 1 December 1963
30 (New Orleans, Louisiana, 21-24 July 1964); 17, Pacific Division (Pacific Grove, California, 18-21 June 1964)	*Annual Reports* 31[#], 102 pages	14 December 1964 (Report for 1967, 34: 84)	LOC = 31 December 1964	Stated Date = 1 December 1964
31 (New York, New York, 20-23 June 1965); 18, Pacific Division (San Diego, California, 24-27 June 1965)	*Annual Reports* 32[#], 122 pages	28 February 1966 (Report for 1967, 34: 84)	LOC = 3 March 1966; UMd = 7 March 1966	Stated Date = 1 December 1965
32 (Chapel Hill, North Carolina, 22-27 August 1966); 19, Pacific Division (Seattle, Washington, 19-22 June 1966)	*Annual Reports* 33[#], 120 pages	28 February 1967 (Report for 1967, 34: 84)	LOC = 8 March 1967; UMd = 16 March 1967	Stated Date = 1 December 1966
33 (Ottawa, Ontario, Canada, 31 July-6 August 1967); 20, Pacific Division (Pacific Grove, California, 28-30 June 1967)	*Annual Reports* 34[#], 119 pages	Mailing Date: 20 March 1968	BMNH = 2 May 1968	Stated Date = 1 December 1967
34 (Corpus Christi, Texas, 15-19 July 1968); 21, Pacific Division (Pacific Grove, California, 19-22 June 1968)	*Annual Reports* 35[#], 97 pages	Mailing Date = 27 December 1968	UMd = 20 January 1969	Stated Date = 1 December 1968
35 (Marinette, Wisconsin, 21-23 July 1969)	*Annual Reports* 36[#], 96 pages	Mailing Date = 19 December 1969	USNM = 13 January 1970	Stated Date = 1 December 1969

Meeting	Publication	Earliest Date of Publication	Later Receipt Dates	Comments
36 (Key West, Florida, 16-20 July 1970)	*Annual Reports* 37#, 112 pages	Mailing Date = 18 February 1971	UMd = 8 March 1971; FMNH = 23 March 1971	Stated Date = 1 December 1970

Bulletin

Meeting	Publication	Earliest Date of Publication	Later Receipt Dates	Comments
37 (Cocoa Beach, Florida, 15-19 July 1971)	*Bulletin*, 70 pages	ANSP = 2 March 1972	FMNH = 8 March 1972	Stated Date = February 1972
38 (Galveston, Texas, 9-14 July 1972)	*Bulletin*, 64 pages	Publication Date = 23 March 1973	USNM = 30 March 1973	
39 (Newark and Greenville, Delaware, 24-28 June 1973)	*Bulletin*, 69 pages	Publication Date = 22 May 1974	ANSP = 28 May 1974; UMd = 28 May 1974	
40 (Springfield, Massachusetts, 3-7 August 1974)	*Bulletin*, 94 pages	Publication Date = May 1975	ANSP = 6 June 1975; UMd = 10 June 1975	
	Index, 1934-1974, [ii] + 57 pp	Probably issued with the preceding in 1975		undated
41 (San Diego, California, 22-26 June 1975)	*Bulletin*, 94 pages + inside back cover	Publication date = 30 January 1976	UMd = 20 February 1976	
42 (Columbus, Ohio, 2-6 August 1976)	*Bulletin*, 89 pages	Publication date = 30 December 1976	UW = 9 February 1977; FMNH = 14 February 1977	
43 (Naples, Florida, 10-15 July 1977)	*Bulletin*, 118 pages	BMNH = 24 April 1978	USNM = 28 April 1978; UMd = 28 April 1978	Membership List = 15 October 1977
44 (Wilmington, North Carolina, 16-21 July 1978)	*Bulletin*, 86 pages	BMNH = 28 March 1979	USNM = 29 March 1979	Membership List = 15 October 1978
45 (Corpus Christi, Texas, 5-11 August 1979)	*Bulletin*, 86 pages	EVC = March 1980	USNM = 20 March 1980; BMNH = 21 March 1980	Membership List = 1 November 1979
46 (Louisville, Kentucky, 19-25 July 1980)	*Bulletin*, 94 pages	USNM = 19 March 1981; BMNH = 19 March 1981	LOC = 21 March 1981	Membership List = 15 October 1980
47 (Ft. Lauderdale, Florida, 19-25 July 1981)	*Bulletin*, 76 pages	BMNH = 15 February 1982	LOC = 23 February 1982; ANSP = 23 February 1982; UMd = 23 February 1982	Membership List = 20 October 1981
48 (New Orleans, Louisiana, 19-23 July 1982)	*American Malacological Bulletin* 1: 136 pages	LOC = 28 June 1983	USNM = 17 July 1983	Stated Date = July 1983 [cover]; May 1983 [1st page and Counts (1988)]
49 (Seattle, Washington, 7-13 August 1983)	*American Malacological Bulletin* 2: 127 pages	BMNH = 30 March 1984	ANSP = 3 April 1984; USNM = 3 April 1984	Stated Date = February 1984
50 (Norfolk, Virginia, 22-27 July 1984)	*American Malacological Bulletin* 3(1): 1-133	BMNH = 18 February 1985	FMNH = 28 February 1985; USNM = 28 February 1985	Stated Date = December 1984

American Malacological Bulletin

Meeting	Publication	Earliest Date of Publication	Latest Date of Publication	Comments
n/a	*American Malacological Bulletin* 3(2): [ii] +135-272	BMNH = 17 July 1985	FMNH = 18 July 1985; LOC = 18 July 1985; UMd = 18 July 1985	Stated Date = June 1985
n/a	*American Malacological Bulletin, Special Edition* 1: 116 pages	(not known; presumably on or after July 1985)	USNM = 7 August 1986 [late]; BMNH = 24 August 1987 [late]	Stated Date = July 1985
51 (Kingston, Rhode Island, 28 July-2 August 1985)	*American Malacological Bulletin* 4(1): 1-147	CAS = 5 March 1986	UW = 14 March 1986	Stated Date = February 1986

Note: the 1985 meeting was the last one to be reported upon in detail in the *American Malacological Bulletin*. For additional information about subsequent AMU/AMS meetings, see Coan and Kabat (1996-present).

American Malacological Bulletin

Publication	Earliest Date of Publication	Latest Date of Publication	Comments
American Malacological Bulletin, Special Edition 2: 239 pages	(not known; presumably on or after June 1986)	FMNH = 1 May 1987 [late]; BMNH = 24 August 1987 [late]	Stated Date = June 1986
American Malacological Bulletin 4(2): 149-247	FMNH = 12 September 1986	AMNH = 15 September 1986	Stated Date = August 1986
American Malacological Bulletin, Special Edition 3: 74 pages	(not known; presumably on or after October 1986)	USNM = 24 April 1987 [late]	Stated Date = October 1986
American Malacological Bulletin 5(1): 1-152	BMNH = 11 February 1987; AMNH = 11 February 1987; FMNH = 11 February 1987	ANSP = 12 February 1987	Stated Date = January 1987
American Malacological Bulletin 5(2): 153-306	BMNH = 14 July 1987	UW = 15 July 1987	Stated Date = June 1987
American Malacological Bulletin 6(1): 1-164	BMNH = 25 February 1988	FMNH = 1 March 1988	Stated Date = January 1988
American Malacological Bulletin 6(2): 165-305	October 1988	USNM = 10 October 1988	Stated Date = October 1988 [as "July 1988" in Counts (1988); corrected to October in *AMB* 7(1): 89
American Malacological Bulletin 7(1): 1-91	BMNH = 24 May 1989; ANSP = 24 May 1989	USNM = 26 May 1989; UMd = 26 May 1989	Stated Date = April 1989
American Malacological Bulletin 7(2): 93-175	UW = 23 March 1990	FMNH = 26 March 1990	Stated Date = February 1990
American Malacological Bulletin 8(1): 1-96	UW = 10 October 1990	BMNH = 12 October 1990	Stated Date = August 1990
American Malacological Bulletin 8(2): 97-182	BMNH = 31 May 1991; ANSP = 31 May 1991	UW = 3 June 1991; UMd = 3 June 1991	Stated Date = April 1991
American Malacological Bulletin 9(1): 1-[104]	BMNH = 30 December 1991	UW = 10 January 1992; ANSP = 11 January 1992	Issue 9(2): 219 gives "December 1991"
American Malacological Bulletin 9(2): 105-219	EVC = August 1992	BMNH = 3 September 1992; ANSP = 3 September 1992	Page 219 gives "August 1992"
American Malacological Bulletin 10(1): 1-[112]	USNM = 18 February 1993	ANSP = 19 February 1993	Issue 10(2): 295 gives "February 1993"

Publication	Earliest Date of Publication	Latest Date of Publication	Comments
American Malacological Bulletin 10(2): iv + 113-295	ANSP = 8 December 1993	USNM = 9 December 1993	Preface gives "July 1993"; page 295 gives "November 1993"
American Malacological Bulletin 11(1): iii + 1-[86] pages	USNM = 30 December 1994; ANSP = 30 December 1994		Page iii gives "December 1994," as does issue 11(2): 211
American Malacological Bulletin 11(2): ii + 87-[212]	ANSP = 8 September 1995	USNM = 11 September 1995	Page 211 gives "August 1995"
American Malacological Bulletin 12(1-2): ii + 1-[156]	AMNH = 24 January 1997	ANSP = 28 January 1997	Page 155 gives "September 1996"; issue 13(1/2): 154 gives "October 1996"; spine gives "1996"
American Malacological Bulletin 13(1/2): ii + 1-[156]	AMNH = 10 January 1997	ANSP = 13 January 1997	Page 154 gives "December 1996"; spine gives "1996"
American Malacological Bulletin 14(1): i + 1-86	ANSP = 5 January 1998	FMNH = 7 January 1998; LOC = 8 January 1998	Issue 14(2): 234 gives "December 1997"
American Malacological Bulletin 14(2): ii + 87-234 pages	LOC = 25 January 1999	AMNH = 28 January 1999	Stated Date "December 1998"
American Malacological Bulletin 15(1): iii + 1-111	ANSP = 15 November 1999	AMNH = 16 November 1999	Stated Date "1999"; issue 15(2): 208 gives "October 1999"
American Malacological Bulletin 15(2): i + [113]-208	BMNH = 22 January 2001	AMNH = 29 January 2001	Page 208 gives "December 2000"
American Malacological Bulletin 16(1/2): iii + 1-266 pages	EVC = 7 November 2001		Page 266 gives "September 2001"
American Malacological Bulletin 17(1/2): iii + 1-165	BMNH = 26 February 2003	ANSP = 3 March 2003; AMNH = 4 March 2003	Stated Date = "December 2002"; to be mailed January 2003 [e-mail, 3 January 2003 from Á. Valdés to R. Bieler]
American Malacological Bulletin 18(1/2): i + 174 pages	7 May 2004		Publication date given on front cover
American Malacological Bulletin 19(1/2): i + 150 pages	14 October 2004		Publication date given on front cover
American Malacological Bulletin 20(1/2): ii + 165 pages	27 April 2005		Publication date given on front cover
American Malacological Bulletin 21(1/2): i + 131 pages	9 February 2006		Publication date given on front cover
American Malacological Bulletin 22(1/2): v + 176 pages	26 March 2007		Publication date given on front cover

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How to Collect Shells

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Taxonomic occurrences of gastropod spermatozeugmata and non-stylommatophoran spermatophores updated

Robert Robertson*

Department of Malacology, The Academy of Natural Sciences of Philadelphia, 1900 Benjamin Franklin Parkway,
Philadelphia, Pennsylvania 19103-1195, U.S.A., hhandrrconch@aol.com

Abstract: Spermatozeugmata, not to be confused with spermatophores, that also transfer sperm, are compound structures (parasperms with attached eusperms) known only in certain "mesogastropods": Loxonematoidea? (Abyssochrysidae), Littorinoidea (Littorinidae), Triphoroidea (Triphoridae and Cerithiopsidae), Tonnoidea (Ranellidae), Janthinoidea (Epitoniidae and Janthinidae), and doubtfully Cypraeoidea (Cypraeidae). This pattern of taxonomic occurrence does not match that of any other character known, their morphology is diverse, and it is concluded that the spermatozeugmata in these taxa are not all homologous and that, like spermatophores, they have evolved repeatedly. Littorinid spermatozeugmata have frequently been studied after fixation and shrinkage of the parasperms ("nurse cells"), during which the eusperms drop off. Spermatozeugmata are not a synapomorphy linking the Triphoroidea and Janthinoidea. Records of spermatophores (except in the "pulmonate" suborder Stylommatophora) since Robertson (1989) are updated.

Key words: sperm transfer, inferred homoplasy, euspermatozoa, paraspermatozoa, nurse cells

SPERMATOZEUGMATA

Spermatozeugmata (singular: spermatozeugma) are not to be confused with spermatophores (Robertson 1989) although both transfer sperm. Spermatozeugmata are of intracellular origin and consist of single paraspermatozoa (Hodgson 1997, Buckland-Nicks *et al.* 2000) with numerous euspermatozoa (fertile sperm) attached externally by their acrosomes. Parasperm are also called apyrene sperm and nurse cells. They are known only in "prosobranchs", but do not all become spermatozeugmata. Spermatophores are secreted extracellularly and contain the eusperms. Spermatophores occur sporadically in many major groups within all gastropods, including many stylommatophorans. Spermatozeugmata are even more sporadic and are known only in certain "mesogastropods":

Incertae sedis (Loxonematoidea?): Abyssochrysidae: *Abyssochrysos* (Healy 1989).

Littorinoidea: Littorinidae: *Littoraria* (Reinke 1911, as "*Littorina*"), *Littorina*, *Littoraria* (Reinke 1912, latter as "*Littorina*"), *Littorina* (Ankel 1930: 599, 600; Linke 1933), *Littoraria* (Woodard 1942a, 1942b, Lenderking 1954, all as "*Littorina*", latter: nurse cell as "spermatophore"), *Melarhaphe* (Battaglia 1952, as "*Littorina*"), *Littoraria* (Marcus and Marcus 1963, as "*Littorina*"), *Bembicium* (Bedford 1965), *Cenchritis* (as "*Tectarius*"), *Tectarius* (as "*Echininus*"), *Littoraria* (as "*Littorina*"), *Nodilittorina* (in part as "*Littorina*" (all Borkowski 1971), *Littorina* (Buckland-Nicks 1973, Buckland-Nicks and Chia 1977), *Nodilittorina* (Jordan and Ramorino 1975, as "*Littorina*"), *Littoraria* (Reid 1986, Healy and Jamieson 1993, Buckland-Nicks *et al.* 2000).

Triphoroidea [including "Cerithiopsoidea"]: Triphoridae: *Triphora* (Houston 1985), *Viriola* (Healy 1987, 1990). Cerithiopsidae: *Cerithiopsis* (Fretter and Graham 1962, Houston 1985), *Seila* (Houston 1985, Healy 1990).

Cypraeoidea?: Cypraeidae?: *Erronea?* (Healy 1986a, as "*Cypraea*").

Tonnoidea: Ranellidae: *Fusitriton* (Buckland-Nicks *et al.* 1982).

Janthinoidea ["Epitonioidea"]: Epitoniidae: *Epitonium* (Ankel 1926, 1938: 6-9, 1958, all as "*Scala*", Fretter 1953, as "*Clathrus*"), *Opalia* (Bulnheim 1962a, 1962b), *Epitonium* (Nishiwaki 1964, Tochimoto 1967, Bulnheim 1968, Nishiwaki and Tochimoto 1969), *Opalia*, *Epitonium* (Melone *et al.* 1978, 1980, latter as "*Scala*"), *Epitonium* (Robertson 1983a, 1983b, Collin 2000, as "*Nitidiscala*"). Janthinidae: *Janthina* (Ankel 1926, 1930, Laursen 1953, Graham 1954, Wilson and Wilson 1956, as "*Ianthina*"). Curiously, two epitoniid species have dimorphic spermatozeugmata (Nishiwaki and Tochimoto 1969).

The all-"mesogastropod" taxonomic pattern of occurrence of spermatozeugmata is non-congruent with any other character known. Robertson (1985, 1989: table 1) and Collin (1997, 2000) reported the congruent occurrences of up to five non-homologous characters, suggesting that the taxa are related. The non-congruence of spermatozeugmata and their varied morphologies suggest that they are not homologous between the superfamilies listed above. The same descriptive name in different taxa does not make a character homologous. The compound origin and nature of spermatozeugmata could well have originated by homoplasy. The Triphoridae appear to have both spermatozeugmata and spermatophores, perhaps in different genera, but this needs confirmation.

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* Current address: 510 Homestead Ave., Haddonfield, New Jersey 08033, U.S.A.

The distinctive nurse cells of littorinids are believed to be parasperms. They are characteristic in being spherical to oblong, with or without projecting rods. In the *Littoraria* subgenus *Palustorina* the nurse cells are elongate or fusiform, having a pseudotrich. Reid has not seen and reported attached eusperms since 1986, perhaps because since then he has consistently studied littorinid parasperms after fixation and shrinkage. As Reinke (1912), Woodard (1942b), Marcus and Marcus (1963), Jordan and Ramorino (1975), and Healy and Jamieson (1993) have observed, the acrosomes are attached weakly and the eusperms drop off easily. Perhaps all littorinids have these evanescent "spermatozeugmata". A cypraeid (*Erronea*) appears to have dimorphic parasperms: vermiform ones as well as spherical, littorinid-like "nurse cells." Eusperms are semi-attached only to the latter, and these "could be considered a form of 'spermatozeugmata'" (Healy 1986a).

Healy (1987) stated that the spermatozeugmata of *Viriola* (Triphoridae) are "very mobile", but in 1990 he stated that they and those of *Seila* (Cerithiopsidae) are "capable of only slow movement." Original observations on the behavior of living janthinoidean spermatozeugmata are included in Wilson and Wilson (1956), Nishiwaki and Tochimoto (1969), Melone *et al.* (1980), and Robertson (1983a). Uniformly, there seems to be pseudocopulation. They are not "vigorously mobile," swimming with "considerable speed" on their "relatively long journeys."

Fretter (1953), Healy (1986b, 1987, 1990, 1994), and Nützel (1998) believed that because of their spermatozeugmata the Triphoridae and Cerithiopsidae are related to the Janthinoidea. They are similar in being large and containing numerous axonemes, but otherwise they are morphologically different in the three groups. Nützel (1998: 2) went so far as to suggest that spermatozeugmata are the "most convincing synapomorphy." Overlooking Nützel's monograph, Collin (2000) reviewed most of the characters traditionally used to support the "Ptenoglossa" (including the Eulimidae but excluding the Architectonicidae, neither of which has known spermatozeugmata). There appear to be no other possible synapomorphies. Other literature on the Triphoroidea and Janthinoidea bears this out: Pruvot-Fol (1925, 1952), Johansson (1947, as "*Scala*", 1953), Risbec (1953), Graham (1954), Marcus and Marcus (1963), Houston (1985), Houbrick (1987), and Collin (2004). "Ptenoglossan" radulae are diverse morphologically. If there is only one supposed "synapomorphy" linking two superfamilies, the validity of it may be questioned. Thus, I agree with Ponder (1998) that it is improbable that Triphoroidea and Janthinoidea are closely related.

SPERMATOPHORES

Records in non-stylommatophorans since Robertson (1989):

Neritoidea: Neritiliidae: *Pisulina* (Kano and Kase 2002), *Neritilia* (Kano *et al.* 2001, Kano and Kase 2003). Neritidae: *Clithon, Neritina* [as "*Neripteron*" and "*Vittina*"] (Starmühlner 1970), *Neritina, Septaria* (Starmühlner 1974), *Clithon, Neritina, Septaria* (Starmühlner 1976), *Clithon, Neritina* (Starmühlner 1983, 1984), *Nerita, Neritilia, Neritina, Puperita* (Starmühlner 1988), *Septaria* (Haynes and Wawra 1989), *Nerita, Neritina* (Houston 1990), *Theliostyla* (Zehra and Perveen 1991), *Nerita, Puperita, Clithon, Neritina, Septaria, Neritilia* (Starmühlner 1993), *Nerita* (Sasaki 1998), *Septaria* (Haynes 2001), *Septaria, Neritina* (Haynes 2005). Phenacolepadidae: *Cinnalepeta?* (Sasaki 1998).

Campaniloidea: Plesiotrochidae: *Plesiotrochus* (Houbrick 1990). [Placement: Healy 1993].

Cerithioidea: Cerithiidae: *Bittium? Bittiolum* (Houbrick 1993), *Cerithium* (Houbrick 1992). Dialidae?: *Diala?* (Ponder 1991). Melanopsidae (as "Thiaridae; Melanopsinae"): *Faunus* (Houbrick 1991b). Paludomidae (including Thiaridae, *s. l.*): *Lavigeria* (Michel 1995), *Tanganyicia* (West 1997, Strong and Glaubrecht 2002), *Tiphobia, Paramelania, Limnotrochus, Chytra, Tanganyicia, Stanleya, Mysorelloides, Reymondia, Lavigeria, Spekia, Stormsia* (Glaubrecht and Strong 2004). Pleuroceridae: *Elimia* (Jones and Branson 1964, as "*Mudalia*"), *Semisulcospira* (Nakano and Nishiwaki 1989). Potamididae: *Terebralia* (Houbrick 1991a). Scaliolidae: *Finella* (Ponder 1994). Thiaridae, *s. l.*: *Vinundu* (Michel 2004). Thiaridae, *s. s.*: *Thiara* (Glaubrecht and Strong 2004). Turritellidae: *Turritella* (Kennedy 1995).

Pterotracheoidea: Atlantidae: *Atlanta* (Jamieson and Newman 1989). Carinariidae: *Pterosoma* (Lalli and Gilmer 1989). The "spermatophores" in Atlantidae reported by Tesch (reference in Robertson 1989) are actually the egg cases of a pleustonic insect, *Halobates* (Seapy 1996).

Vermetoidea: Vermetidae: *Dendropoma, Serpulorbis, Vermetus* (Calvo and Templado 2005).

Pyramidelloidea: Pyramidellidae: *Pyramidella?* (Ponder 1987), *Boonea* (Wise 2001), *Fargoa* (Robertson 1996), *Iolaea* (Hori and Kuroda 2001), *Odostomella* (Schander *et al.* 1999), *Parthenina* (Hori and Kuroda 2002).

Cavolinioidea: Cavoliniidae: *Diacria* (Lalli and Gilmer 1989). Limacinidae: *Limacina* (Lalli and Gilmer 1989).

Clionoidea?: Pneumodermatidae?: *Crucibranchaea?* (Lalli and Gilmer 1989).

Hedylopsoidea: Parhedylidae: *Pontohedyle, Unela* (Poizat 1989).

Aeolidioidea: Aeolidiidae: *Aeolidiella* (Haase and Karlsson 2000).

Spermatophore presence can be difficult to ascertain, and a "spermatophore bursa" in a pallial oviduct may not always receive one (despite my assumption to the contrary in 1989: 362, A7). In Robertson (1989) I reported spermatophores in the cerithioidean Litiopidae, but Houbrick observed only bursae in them. Glaubrecht and Strong (2004) inferred the spermatophore-forming organ in male paludomid cerithioideans.

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A developmental perspective on evolutionary innovation in the radula of the predatory neogastropod family Muricidae*

Gregory S. Herbert[1], Didier Merle[2], and Carlos S. Gallardo[3]

[1] Department of Geology, University of South Florida, Tampa, Florida, U.S.A., gherbert@cas.usf.edu

[2] Unité de Paléontologie, Département Histoire de la Terre, Muséum national d'Histoire naturelle, UMR 5143 CNRS, 8, rue Buffon, 75005 Paris, France, dmerle@mnhn.fr

[3] Instituto de Zoologia, Universidad Austral de Chile, Casilla 567, Valdivia, Chile, cgallard@uach.cl

Abstract: The neogastropod family Muricidae includes a diverse set of radular bauplane, including a beaked, three-dimensional form, a flattened-pentacusped form, and a third "dagger" type in which the central rachidian cusp is massive and elongate. Examination of the radular ontogenies of representatives of five muricid subfamilies reveals that several species undergo changes in radular form during ontogeny on a scale comparable to the evolutionary differences between higher taxa. The species *Concholepas concholepas* (Bruguière, 1789) (Rapaninae) and *Trophon geversianus* (Pallas, 1774) (Trophoninae) begin ontogeny with a three-dimensional rachidian characteristic of the Ocenebrinae or Muricopsinae but end with the dagger rachidian typical of their respective subfamilies. Young individuals of *Vitularia salebrosa* (King and Broderip, 1832) (Muricopsinae?) also have a three-dimensional rachidian but shift to a double-dagger morphology by adulthood. *Chicoreus* (*Phyllonotus*) *pomum* (Gmelin, 1791) (Muricinae) has a typical flattened muricine rachidian as an adult but possesses a "buccinoid"-like rachidian just after hatching. *Urosalpinx cinerea* (Say, 1822) (Ocenebrinae), was unique among the species examined in exhibiting no ontogenetic changes in radular form. The occurrence of two radular bauplane within the same individual snail during ontogeny suggests great potential for rapid, convergent evolution of adult features through simple changes in developmental timing. A three-dimensional rachidian, for example, could be retained into adulthood through paedomorphosis in any lineage possessing the three-dimensional-to-dagger ontogeny. Systematic assignments of muricids based solely on radular features should be reexamined.

Key words: muricid, rachidian, bauplan, ontogeny, heterochrony

The radulae of a number of gastropod species undergo small to large-scale changes in the number, type, and structural complexity of teeth between the pre-metamorphic larval stage, when the radula first forms, and maturation (references in Fujioka 1984a, Page and Willan 1988, Nybakken 1990, Warén 1990). A controversial but long-standing idea is that such changes in development play a dominant role in the evolutionary origins of new characters, or innovations, and, hence, in the origin of higher taxa (reviewed in Gould 1977). Rather than requiring widespread alteration of structural genes controlling morphology, innovation may derive simply through small-scale changes in regulatory genes controlling the rate and/or timing of development, *i.e.*, heterochrony. Size changes associated with heterochronic evolution may also provide a catalyst for extensive innovation throughout the organism. Changes in skeletal structure, for example, often evolve to compensate for the detrimental by-products of developmental miniaturization (see references in Hanken 1985, Hanken and Wake 1993).

With few exceptions (*e.g.*, Guralnick and Lindberg 1999), however, molluscan biologists have yet to investigate radular evolution from the perspective of development. In the present study, we document radular ontogenies in several taxa belonging to the predatory gastropod family Muricidae and examine the possibility that the evolution of developmental timing has played a central role in the repeated origins of subfamily-level radular bauplane within the Muricidae. Phylogenetic studies are necessary to test the hypothesis that heterochronic mechanisms have been involved in the evolution of any particular structure, but ontogenetic analyses presented herein are a necessary first step.

BACKGROUND

Radular bauplane in the Muricidae

Two nomenclatural schemes have been utilized in the past to describe the basic structural types (referred to throughout this paper as "bauplane") of the muricid rachidian teeth and to delineate the muricid subfamilies. The first system, established by Arakawa (1962, 1964, 1965) and Wu (1965, 1968, 1973), groups muricids according to the number of cusps on the rachidian tooth. These workers subdivided muricid radulae into two classes—a complex "pentacusped" rachidian having a central cusp, two lateral cusps, and two marginal cusps, and a simplified "tricusped" rachidian having only the central and two lateral cusps (Fig. 1). Fujioka (1985a) later added a third class for taxa having a "monocusped" rachidian (*i.e.*, only a central cusp) and recognized a number of intermediate classes as well. As noted by Kool (1987), however, this system suffered from the rather arbitrary manner in which it was applied and is no longer used. For example, some authors counted only major cusps but others counted both cusps and denticles. The variable size, position, and number of cusps and denticles in different muricids make this system impossible to apply unambiguously (Kool 1987).

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An alternative system, built upon in this paper, was developed by Vokes (1971), Radwin and D'Attilio (1971, 1976), and D'Attilio (1980, 1991) and is based largely upon variation in the morphology of the most prominent structure on the rachidian—the central cusp—rather than the number of cusps on the tooth. In the "flattened" type, the rachidian is broad, with all the cusps lying in the same plane and the central cusp being slightly longer than either of the laterals (Fig. 2M). This type occurs in almost all species presently assigned to the subfamilies Muricinae, Typhinae, Tripterotyphinae, and Haustrinae, as well as in many species currently assigned to the Trophoninae and the *Murexsul-Muricopsis* genus group of the Muricopsinae (Radwin and D'Attilio 1971, 1976, Vokes 1971) (Figs. 2A-L). Because the Muricinae predate all other muricid subfamilies by at least 20 million years (see Vokes 1971, 1990, 1992, 1994, Garvie 1991, 1992, Marko and Vermeij 1999, Merle 1999, Vermeij and Carlson 2000), and because the anatomical condition of the Muricinae is likely primitive within the Muricidae (Harasewych 1984), the flattened rachidian type of muricines and other muricids is presumably the plesiomorphic condition for the family.

In a second type, which Vokes (1971) referred to as "three-dimensional" or "3-D," the rachidian base is narrow (Fig. 3J) and rectangular, with a short, beak-like central cusp that projects up to 90 degrees away from the rachidian base and up to 45 degrees away from either lateral cusp (Fig. 3I). Vokes (1971) and Radwin and D'Attilio (1971) have used terms such as "triangular harrow," "cowl-like," and "fang-like" to describe this type as well. A 3-D rachidian type characterizes Vokes' (1971) *Murexiella* genus group of the Muricopsinae (= *Favartia/Pygmaepterys* subclade of Merle and Houart 2003), some species of *Muricopsis* (see Radwin

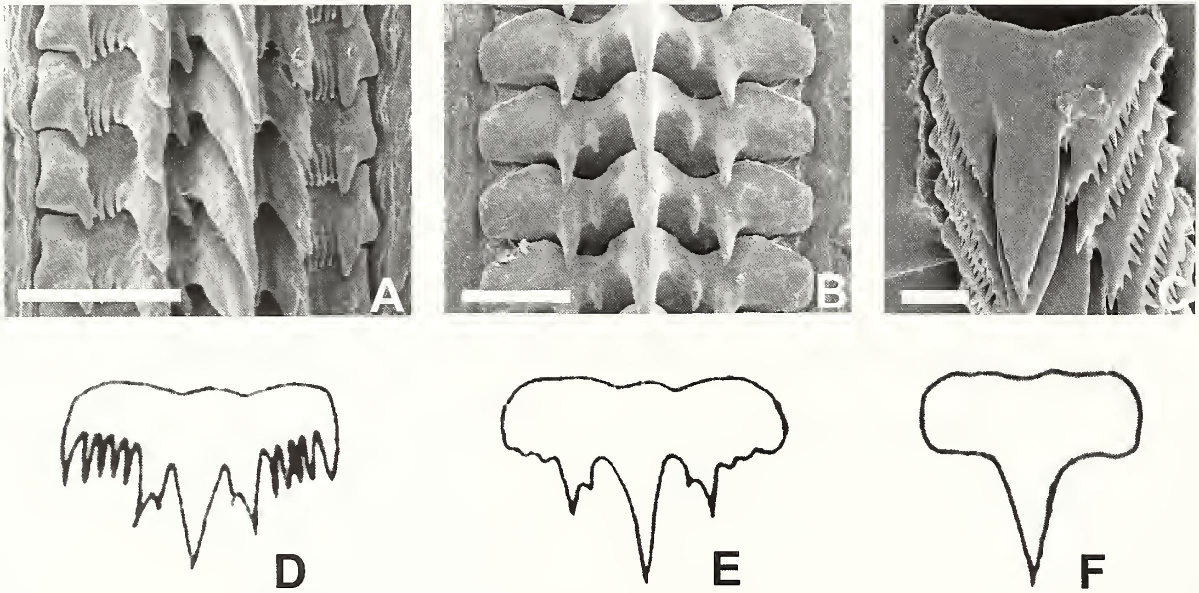

Figure 1. Pentacusped, tricusped, and monocusped rachidian bauplane of Arakawa (1962, 1964, 1965), Wu (1965, 1968, 1973), and Fujioka (1985a). A. The pentacusped rachidian characterized by the rapanine *Neothais harpa* (Conrad, 1837), locality: Maui, Hawaiian Islands, scale bar = 50 µm. B. The tricusped rachidian characterized by the ergalataxine *Cronia crassulnata* (Hedley, 1915), locality: Gulf of Carpentaria, northern Australia, scale bar = 50 µm. C. The monocusped rachidian characterized by the rapanine *Drupella elata* Blainville, 1832, Kauai, Hawaiian Islands, scale bar = 20 µm. D-F, penta-, tri-, and monocusped rachidia, modified from Fujioka (1985a, fig. 8).

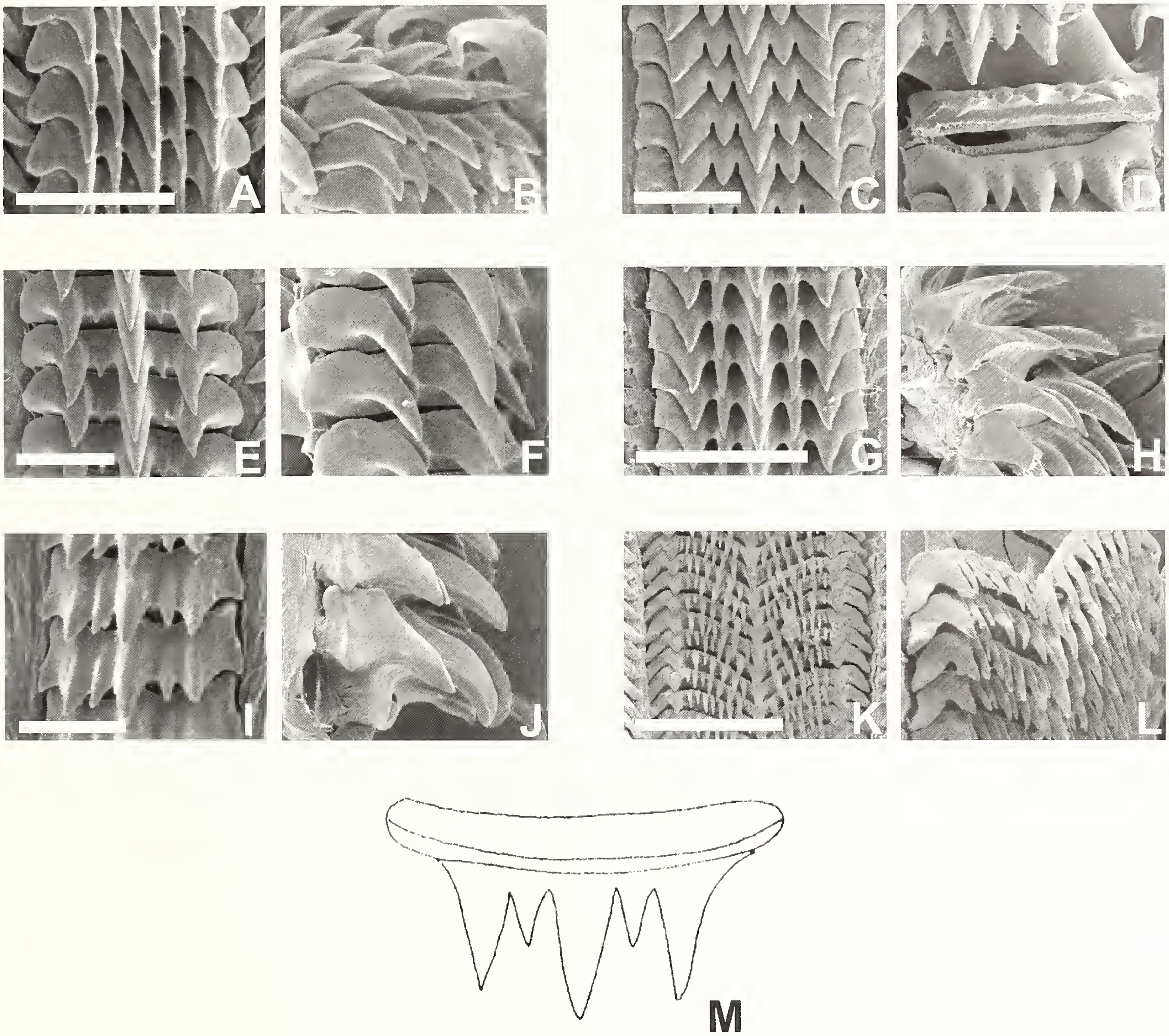

Figure 2. The "flattened" rachidian bauplan. A-B. The muricine *Aspella indentata* Carpenter, 1857, locality: Mira Mar, north of Manzanillo, Mexico, scale bar = 50 μm. C-D. The muricine *Murex brevispina* var. *macgillivrayi* Dohrn, 1862, locality: Papau New Guinea, scale bar = 100 μm. E-F. The "trophonine" *Xymenopsis buccineus* (Lamarck, 1816), locality: Tierra del Fuego, Argentina, scale bar = 50 μm. G-H. The haustrine *Haustrum haustorium* (Gmelin, 1791), locality: New Zealand, scale bar = 100 μm. I-J. The muricopsine *Murexsul octagonus* Quoy and Gaimard, 1833, locality: New Zealand, scale bar = 50 μm. K-L. The typhine *Typhisala grandis* (A. Adams, 1855), locality: Golfo de Tehuantepec, Mexico, scale bar = 50 μm. M. The muricine rachidian bauplan, modified from Vokes (1971, fig. 2a).

and D'Attilio 1976), and the *Ocenebra-Ocinebrina* genus group of the Ocenebrinae (*sensu* Vermeij and Vokes 1997). Other muricid taxa that possess a beak-like central cusp include the putative-muricopsines *Vitularia* Swainson, 1840; *Acanthotrophon* Hertlein and Strong, 1951; and *Bizetiella* Radwin and D'Attilio, 1972; and the muricine *Chicopinnatus laqueatus* (Sowerby, 1841) (Figs. 3A-H).

In addition to these two general categories of rachidia, we recognize a third—a "dagger" rachidian—for muricids having a flattened rachidian but modified with a more massive and considerably more elongate central cusp and much weaker lateral and marginal cusps (Fig. 4I). Fujioka's (1985a) "monocusped" rachidian (Figs. 1C, 1F) is an extreme form of this third type. Muricids possessing a dagger-type rachidian include most rapanine and ergalataxine species as well as the "*Thais*-like" ocenebrines (*e.g.*, *Nucella*

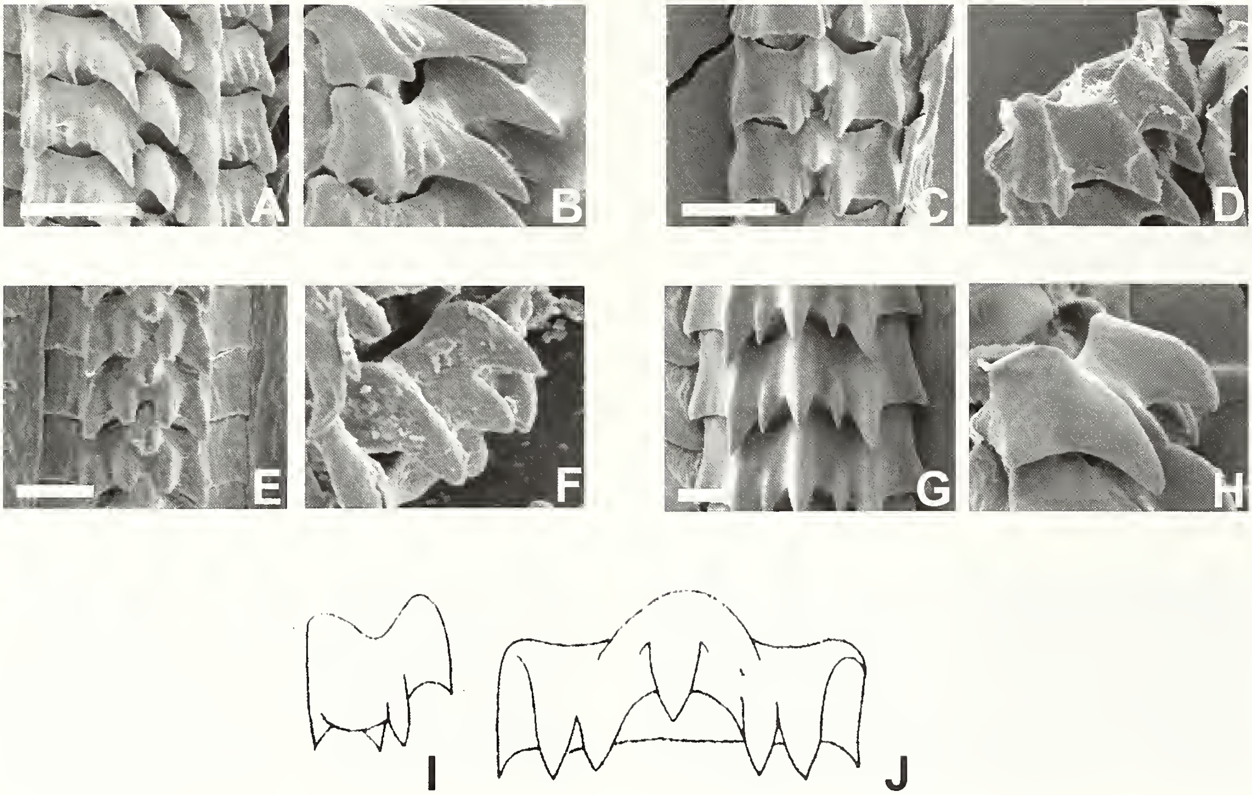

Figure 3. The "three-dimensional" rachidian bauplan. A-B. The ocenebrine *Urosalpinx perrugata* (Conrad, 1846); locality: St. Joseph's Bay, Florida, scale bar = 50 µm. C-D. The muricopsine *Caribiella alveata* (Kiener, 1842), locality, Discovery Bay, Jamaica, scale bar = 20 µm. E-F. The muricopsine *Acanthotrophon sorenseni* Hertlein and Strong, 1951, locality: Gulf of California, Mexico, scale bar = 20 µm. G-H. The muricine *Chicopinnatus laqueatus* (Sowerby, 1841), locality: Orote Point, Guam, Oceania, scale bar = 20 µm. I-J. The three-dimensional rachidian bauplan, modified from Vokes (1971, fig. 2b).

Röding, 1798; *Acanthina* Fischer de Waldheim, 1807; etc., see Vermeij and Vokes 1997), *Trophon geversianus* (Pallas, 1774) (type species of the type genus of the Trophoninae), and several problematic South American genera (*e.g.*, *Chorus* Gray, 1847; *Xanthochorus* Fischer, 1884) (Figs. 4A-H).

Researchers have long relied upon these bauplane to assign species to subfamilies and reconstruct muricid phylogeny based on the assumption that the radula is a conservative character complex relative to features of the shell and operculum (Vokes 1964, 1971, Radwin and D'Attilio 1971, 1976, Houart 1992; see additional references in Kool 1987). Vokes (1971), for example, regarded the subfamily Ocenebrinae as phylogenetically nested within the Muricopsinae on the basis of both groups sharing a 3-D rachidian, despite the fact that other morphological features, such as those of the early shell whorls and operculum, support different relationships (Vokes 1971, Kool 1993a, Merle 1999, Vermeij and Carlson 2000). Using the same logic, Radwin and D'Attilio (1976) assigned the genus *Vitularia* to the Muricopsinae, although shell and opercular characters are difficult to reconcile with this classification (see Vokes 1967, 1977, 1986). Most recently, Bouchet and Houart (1996) reassigned the muricine *Chicoreus gubbi* (Reeve, 1849) to the Ocenebrinae as a new genus *Chicocenebra* Bouchet and Houart, 1996 based solely on its having a 3-D rachidian, even though its classification as a member of the Muricinae had not been questioned previously when only the shell morphology of this species was known.

Previous studies of radular development and evolution in muricids

If bauplan-level transformations in the muricid radula can occur as a result of small-scale changes in developmental timing, then radular features may be less conservative and, thus, less phylogenetically informative, than is currently thought. At present, the best answer to this question comes from a series of seminal papers by Fujioka (1982, 1984a, 1984b, 1985a, 1985b) on the radulae of the subfamilies Rapaninae and Ergalataxinae. Fujioka found that the lateral cusps, marginal cusps, and intermediate and marginal den-

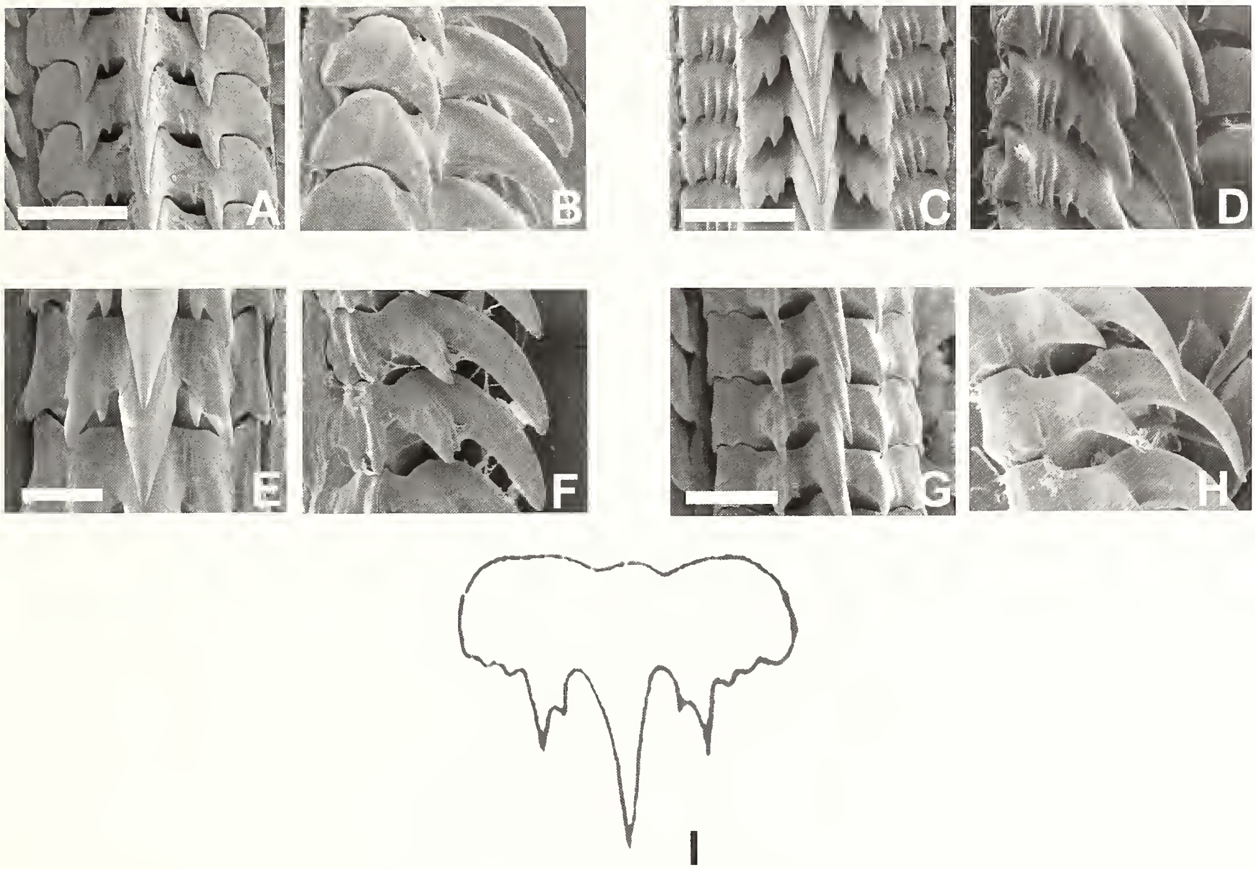

Figure 4. The dagger rachidian bauplan. A-B. The ergalataxine *Cronia (Cronia) avellana* (Reevem, 1846), locality: western Australia, scale bar = 100 μm. C-D. The rapanine *Agnewia tritoniformis* (Blainville, 1833), locality: Manly, New South Wales, Australia, scale bar = 50 μm. E-F. The ocenebrine *Nucella ostrina* Gould, 1852, locality: Monterey, California, scale bar = 20 μm. G-H. The ergalataxine? *Xanthochorus cassidiformis* Blainville, 1824, locality: Metri Bay, Chile, scale bar = 50 μm. I. The ergalataxine rachidian bauplan, modified from Fujioka (1985a, fig. 8).

ticles in many species of these two subfamilies become progressively atrophied during ontogeny, while the central cusp becomes longer and its base becomes wider.

Rapanines, however, begin ontogeny with a pentacusped rachidian and typically end at the pentacusped or tricusped stage, whereas many of the ergalataxines studied begin ontogeny at the tricusped stage and end with a tricusped or monocusped rachidian. Under the assumption that new characters, such as atrophication, are only added to the end of ontogeny, Fujioka reasoned that the less atrophied pentacusped rachidia of rapanines is the relatively primitive condition and that the ergalataxine condition evolved by peramorphic heterochrony (extended atrophication) of this ancestral ontogeny. Phylogenies published recently for the Rapaninae generally support this evolutionary scenario with ergalataxines depicted as a nested clade within the Rapaninae (*i.e.*, Kool 1993a, Vermeij and Carlson 2000, but see Tan 2003).

More recently, DiSalvo (1988) and DiSalvo and Carriker (1994) documented ontogenetic changes in the rachidian tooth morphology of the rapanine muricid *Concholepas concholepas* (Bruguière, 1789). This species was found to change from a 3-D rachidian in early post-metamorphic animals to a dagger-type rachidian in small sub-adults. Although these workers did not comment on the evolutionary significance of their observations or document the exact size at which this transition occurs, it suggests to us that the 3-D and dagger rachidia in adults could potentially evolve rapidly from one to the other through heterochronic processes.

Focus of the present study

A 3-D rachidian was reported in none of the many juvenile rapanine species studied by Fujioka, which makes its more recently reported occurrence in the rapanine *Concholepas* suspect. Thus, the initial goal of the present study was to test the results of DiSalvo and Carriker by collecting new early post-larval specimens for *Concholepas concholepas*, verifying their taxonomic identity, and re-examining their early stage radulae using SEM.

The second focus of this paper was to examine the ontogenies of muricids outside of the Rapaninae and Ergalataxinae. With the exception of Houart's (1992) illustration of the radular ontogeny of the muricine *Chicoreus (Triplex) torrefactus* (Sowerby, 1841), there have been no attempts to document ontogenetic series in non-rapanine or non-ergalataxine muricids. Our present study of new and previously collected material allows us to examine for the first time the ontogenies of species representing the Trophoninae, Ocenebrinae, a second species of the Muricinae, and a putative member of the Muricopsinae.

The third focus of this study was to investigate whether large-scale morphological shifts occur during the earliest ontogenetic stages of development, *i.e.*, between larval metamorphosis and juveniles around 10 mm shell length. Fujioka examined no radulae from early post-metamorphic stage individuals to small juveniles 3 mm in shell length. For more than half the species Fujioka studied, he examined no juveniles smaller than 10 mm in length, and many of his smallest "juveniles" were greater than 25 mm in shell length. DiSalvo's work, in contrast, suggested that changes during earliest ontogeny (*i.e.*, less than 10 mm shell length) may be substantial (DiSalvo 1988, DiSalvo and Carriker 1994) and possibly be related to changes in mode of predation and feeding behavior that can occur within this size range (see discussion section). This study documents the endpoints of the entire ontogenetic sequences beginning with rachidia in earliest post-metamorphic individuals, material permitting.

MATERIALS AND METHODS

Radula preparation

Radulae were recovered from late juvenile and adult alcohol-preserved and dried specimens by dissolving dissected proboscis tissues in a concentrated solution of potassium hydroxide (KOH) for 1-3 days. Radular ribbons, visible with the naked eye, were collected with forceps, rinsed in a series of hot distilled-water washes, fixed to aluminum tabs with double-sided conductive tape, air dried, and gold coated (40 nm thickness) for scanning electron microscopy.

Radulae of early juvenile snails were recovered from dried and alcohol preserved specimens by gently crushing the larval shells in a shallow petri dish filled with a concentrated solution of KOH. After two hours, the dish was heated to 90°C to reverse any precipitation of KOH crystals that might have formed on the radular ribbon. Because they were too small to be collected with forceps, radulae were removed from the KOH solution using a dropper partially filled with warm distilled water. This was done to dilute the KOH solution collected with the radula and prevent later precipitation of KOH crystals on the radular ribbon during drying. The dilute KOH solution with the radula was then transported by dropper through two rinses of hot distilled water in separate dishes for additional dilution. After two rinses, cleansed radulae were transferred in distilled water by dropper to an aluminum tab coated with double-sided conductive tape and air dried. Aluminum tabs with radulae were gold coated (40 nm thickness). All radulae were examined with an ISI DS-130 scanning electron microscope at the University of California, Davis's Facility for Advanced Instrumentation.

Radula Terminology

Throughout the remainder of this paper, we use the standard terminology illustrated by Radwin and D'Attilio (1976) and Kool (1987, 1993a) to refer to parts of the rachidian radular tooth.

RESULTS

Subfamily RAPANINAE
Concholepas concholepas (Bruguière, 1789)
(Figs. 5A-F)

Material examined

Nineteen juvenile to sub-adult specimens of *Concholepas concholepas* ranging in shell length from 11 to 30 mm were collected in March 2001 from Mehuin, 70 km north of Valdivia, Chile, and placed in 50% ethanol for later dissection. An additional nine early post-metamorphic individuals ranging from 1.7 to 3.9 mm were captured between September 1999 and January 2000 and between January 2000 and August 2000 from artificial collection plates installed at Las Cruces (coast of Santiago Province), Chile, and stored in 50% ethanol.

Ontogeny

In early post-metamorphic juveniles with shell lengths ranging from 1.7 to 3.9 mm, rachidian widths are 20 µm and have a 3-D rachidian morphology (Figs. 5A-D). Most have a single marginal denticle, although the largest individual in this class had two in one marginal area and one in the other (Fig. 5A). The rachidian base end-point is marked by a prominent marginal cusp that runs parallel to the rachidian base. Behind this marginal cusp is a shorter marginal cusp that is poorly developed and bud-like. The two cusps combined loosely resemble the double marginal cusps of the Ocenebrinae.

In young animals with shell lengths between 11 and 13

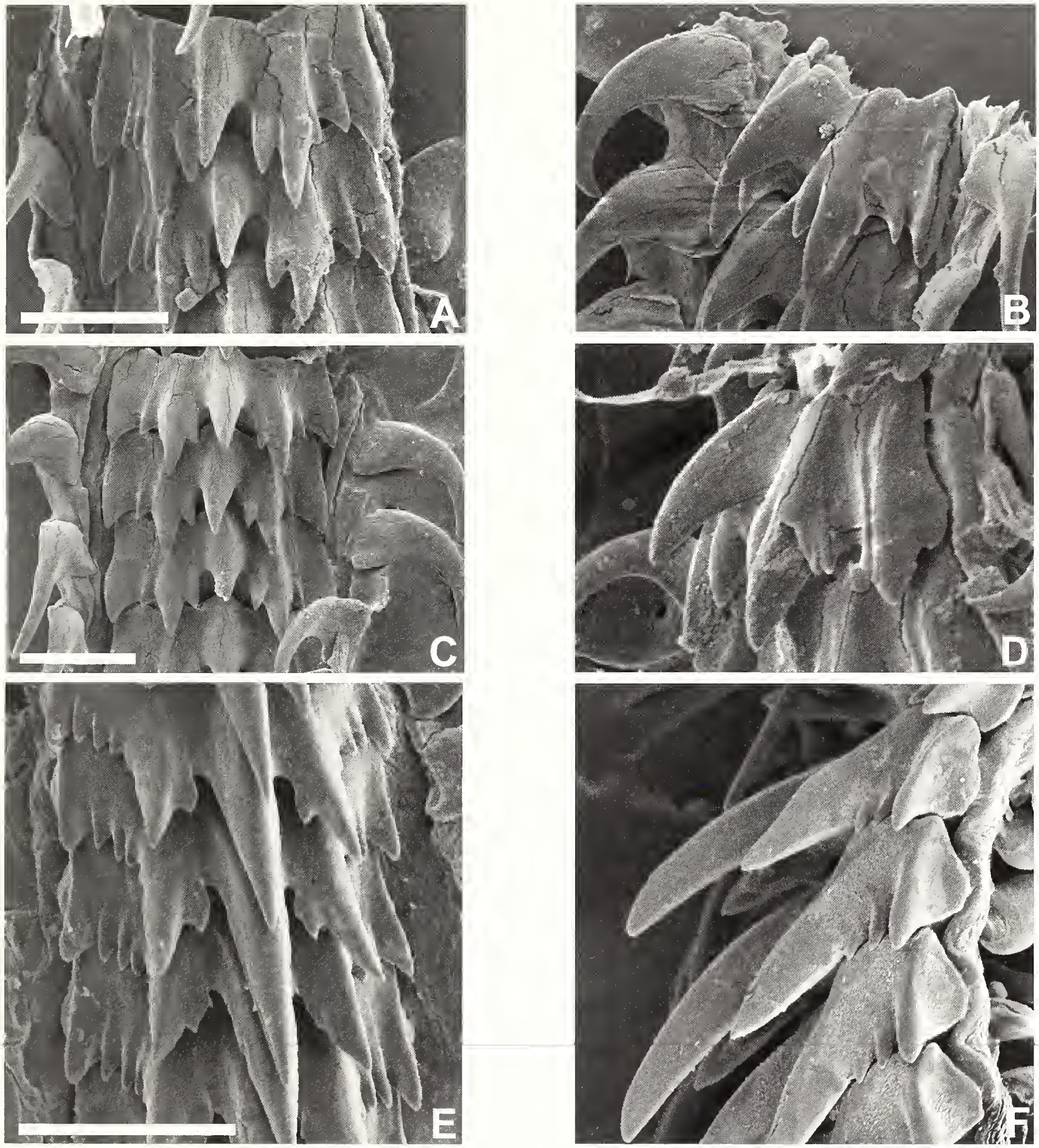

Figure 5. Radular ontogeny of the rapanine muricid *Concholepas concholepas*. A-D. Front and lateral views of rachidia of early post-metamorphic individuals ranging from 1.7 to 3.9 mm in shell length, locality: Las Cruces, Chile, scale bars = 10 µm. E-F. Front and lateral views of rachidia of a small juvenile with shell length of 15 mm, locality: Mehuin, Chile, scale bar = 50 µm.

mm, the rachidian tooth increases to approx. 100 µm in width and assumes the dagger-type morphology (Figs. 5E-F). The lateral cusps are turned outwardly at their distal ends, marginal cusp number increases to two or three, and outer lateral denticles begin to appear. The rachidian base begins to develop a small, rounded lateral extension at both ends, which may be homologous with the bud-like second marginal cusp observed in the early post-metamorphic juveniles. The radular ontogeny of *Concholepas* is essentially stabilized at the dagger-type rachidian by a shell length of 11 mm with little modification afterwards. Large adults may reach shell lengths upwards of 125 mm. Radulae of larger adult animals are figured elsewhere (Kool 1987, 1993b, DiSalvo 1988) for comparison.

Remarks

DiSalvo (1988) and DiSalvo and Carriker (1994) illustrated the rachidian tooth morphology of the pediveliger stage (1.6-1.9 mm shell length) of the rapanine *Concholepas concholepas* from neustonic pediveliger larvae reared from egg capsules captured at sea and hatched in the lab. The present study confirms their data showing this early stage to have a 3-D rachidian. Possession of a 3-D-type rachidian during any stage of ontogeny in a rapanine is unusual because previous studies of radular ontogeny of rapanines and a nested subclade within the Rapaninae, the Ergalataxinae, have reported only the flat rachidian type at any stage of development (Fujioka 1984a, 1984b, 1985a). However, given the size range of juvenile rapanines examined by Fujioka, it is possible that this stage of ontogeny was overlooked.

The transition from 3-D to dagger-type rachidian occurs after metamorphosis (DiSalvo, pers. comm.) and between shell lengths of 4 and 11 mm (this study).

Subfamily TROPHONINAE
Trophon geversianus (Pallas, 1774)
(Figs. 6A-D)

Material examined

Research material for *Trophon geversianus* (Pallas, 1774) was obtained from dried and alcohol-preserved specimens in the personal collection of E. H. Vokes (Tulane University). This material included ten dried pre-hatched juveniles, all less than 2 mm in shell length, removed from a single egg capsule collected at a beach at Río Grande, Tierra del Fuego, Argentina. Although not yet hatched, the animals appear to have undergone metamorphosis, as indicated by the initiation of teleoconch (adult) sculpture. Also sampled were twenty alcohol-preserved adult specimens (all > 30mm) from Bahía El Pescador, south of Puerto Pirámides, in the northeastern part of Golfo Nuevo, Argentina. This collection was also from the collection of E. H. Vokes.

Ontogeny

In post-metamorphic, pre-hatched juveniles of this species, the rachidian tooth is approx. 10 μm in width and has a 3-D morphology (Figs. 6A-B). The intermediate denticle is long and has an attachment site on the rachidian base independent of the lateral cusp but closer to the lateral than the central cusp. The basal region is rectangular and deep, with a strong marginal cusp and a second bud-like cusp closer to the radular ribbon.

Rachidian teeth in adults sampled are approx. 200 μm in width and exhibit a dagger-type morphology (Figs. 6C-D). The intermediate denticle is shorter than in early ontogeny (only one-fifth the height of the lateral cusp) and fused with the lateral cusp instead of having a separate attachment site on the rachidian base. The outer edges of the lateral cusps possess small serrations or outer lateral denticles. The basal end-point is rectangular but shallow and marked by a single marginal cusp. The second bud-like cusp of early ontogeny is obsolete or nearly so.

Remarks

Several authors have published line drawings and scanning electron micrographs of the radulae of adult *Trophon geversianus,* including Radwin and D'Attilio (1976), Kool (1993a), and Pastorino (2002). The present study is the first to document the morphology of the rachidia in early post-metamorphic individuals.

Subfamily OCENEBRINAE
Urosalpinx cinerea (Say, 1822)
(Figs. 7A-D)

Material examined

Specimens of the ocenebrine muricid *Urosalpinx cinerea* were obtained in May 2001 from intertidal barnacle, mussel, and bryozoan-encrusted rocks from a jetty in San Francisco Bay in Burlingame, California, USA. This species is native to the western Atlantic, but was introduced to the eastern Pacific nearly a century ago (Radwin and D'Attilio 1976). Twenty large adults (20-30 mm), including both males and females, were collected at the Burlingame locality and preserved immediately in 75% ethanol for later dissection. Another 10 individuals were placed in a single aquarium with recirculating seawater and provided with barnacles for food. Within days, adult snails deposited egg capsules on tank walls. After approx. six weeks, several hundred hatchlings (1.5-2.0 mm shell length) emerged from the capsules as crawl-away juveniles and began drilling barnacles provided and cannibalizing one another by drilling. Approximately 50 hatchlings were harvested immediately and preserved in 75% ethanol.

Ontogeny

Animals ~2.0 mm in shell length possess rachidia 10 μm in width with a 3-D morphology (Figs. 7A-B). Adult rachidia are larger (approx. 100 μm) but nearly identical in shape (Figs. 7C-D).

Remarks

Radwin and D'Attilio (1976) and Kool (1993b) illustrated the adult radula of *Urosalpinx cinerea*, but there have

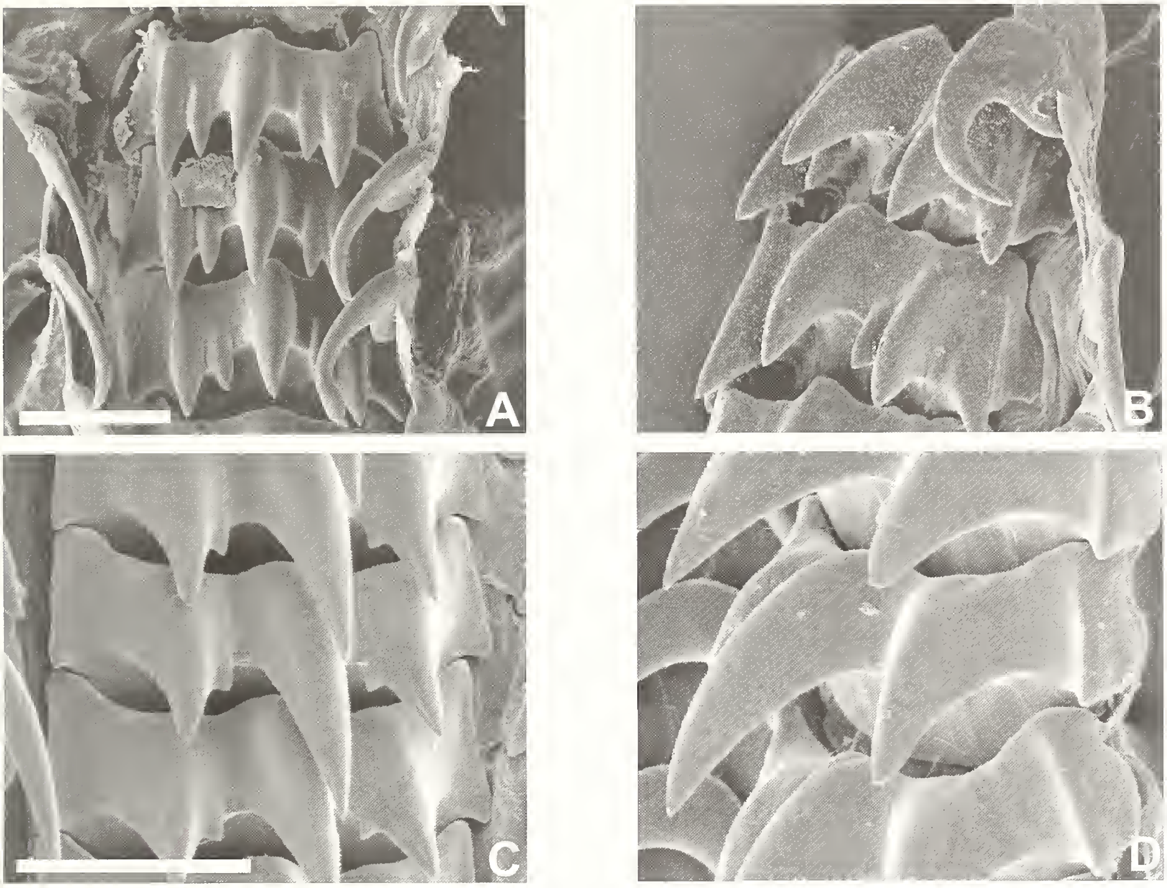

Figure 6. Radular ontogeny of the trophonine muricid *Trophon geversianus*. A-B. Front and lateral views of rachidia of early post-metamorphic (pre-hatched) individuals with shell lengths of 1.5 to 2.0 mm, locality: Tierra del Fuego, Argentina, scale bars = 10 µm. C-D. Front and lateral views of rachidia of adult specimen with shell length of 30 mm, locality: Golfo Nuevo, Argentina, scale bar = 100 µm.

been no studies of radular morphology during early ontogeny. Carriker (1969) figured the mature radula of a subspecies, *Urosalpinx cinerea* var. *etterae* Baker, 1955, including eight scanning electron micrographs of radulae from various angles and two light micrographs of a rasping radula in the process of drilling a shell. The radula of this subspecies appears to differ from that of the nominate form in having one or two extra marginal denticles.

Subfamily MURICOPSINAE?
Vitularia salebrosa (King and Broderip, 1832)
(Figs. 8A-I)

Material examined

One dried juvenile specimen of *Vitularia salebrosa* (9.9 mm shell length) and ten dried adult specimens (30-50 mm shell length) from various tropical eastern Pacific localities were obtained from the personal research collections of E. H. Vokes and G. J. Vermeij. No other specimens of this species were available at the time the study was conducted.

Ontogeny

At the small juvenile stage (one 9.9 mm specimen), the rachidian is just over 5 µm in width and resembles the 3-D type in having a short, beak-like central cusp (Figs. 8A-C). However, the rachidian base is flat rather than rectangular, a condition typically associated with flattened rachidia. Adjacent to the central cusp are three pairs of short, conical "cusps." The intermediate denticle is unusual for the 3-D type in projecting further from the rachidian base than the adjacent "lateral" cusp. The attachment site for the denticle is separate from the lateral cusp as in some muricopsines. The outermost cusp sits far from the margin endpoint, which curves into a pseudo-cusp. The rachidian of a larger specimen (31 mm shell length) differs in having a slightly longer outermost cusp.

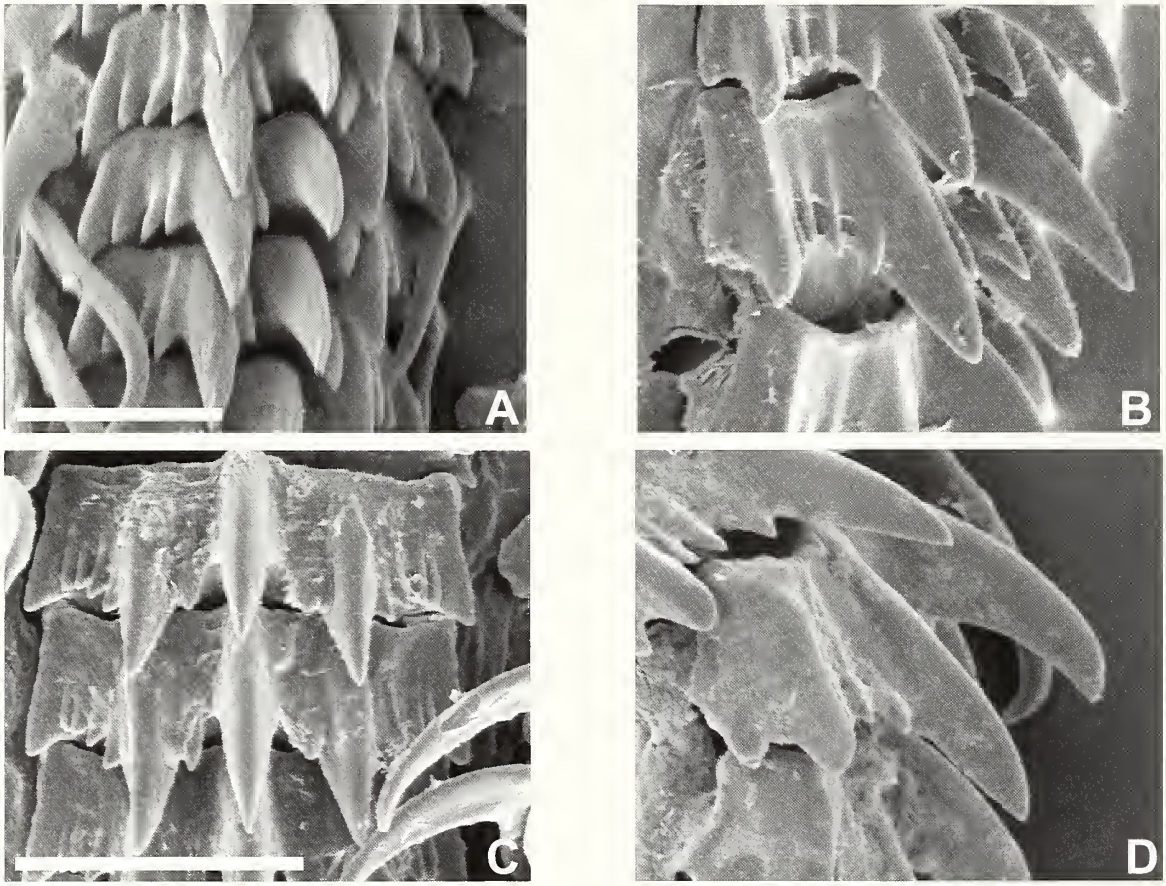

Figure 7. Radular ontogeny of the ocenebrine muricid *Urosalpinx cinerea.* A-B. Front and lateral views of rachidia of early post-metamorphic individuals with shell lengths of 1.5 to 2.0 mm, locality: San Francisco Bay, California, scale bar = 5 μm. C-D. Front and lateral views of rachidia of adult specimen with shell length of 25 mm, locality: San Francisco Bay, California, scale bar = 50 μm.

The largest individuals of *Vitularia salebrosa* available to us (40 to 50 mm specimens) failed to produce a radula in eight of the nine specimens we examined (89%). A single radula from a shell 50 mm in length shows rachidian teeth to be approx. 70 μm in width with long, tusk-like outermost (marginal?) cusps situated far from the base endpoint (Figs. 8G-I). The overall morphology of the central cusp is roughly of the 3-D rachidian type, but the outermost cusps are massive and elongated as in the typical dagger-type rachidian.

Remarks

D'Attilio's (1991) investigation of the radula of *Vitularia salebrosa* showed that this species has at least two different radular morphotypes, including a "normal" rachidian with seven cusps and a second rachidian with only three cusps, which he described as "extremely aberrant resembling nothing else known to me." The latter morphotype has one subobsolete central cusp and two massive, incurved, tusk-like lateral cusps. D'Attilio did not provide information on the sizes of the "normal" and "aberrant" rachidia, but the present data suggest they could represent end-members of a single ontogenetic sequence.

A second "aberrant" feature of the radula of *Vitularia salebrosa* is its occasional absence. D'Attilio (1991) reported that his own efforts to recover a radula from this species were successful only twice out of ten total attempts, which is similar to the success rate of one out of nine attempts reported in this study. This species is parasitic on oysters and attacks by pushing the proboscis between the valve margins, aided initially by drilling (G. S. Herbert and G. P. Dietl, pers. obs.). Attacking oysters at the edge could result in amputation of the proboscis and radular loss when the oyster closes its valves. Another possibility is that the radula is used only to initiate an edge-drilled hole, and afterwards, the animal reabsorbs used teeth and stops forming new ones as it begins a parasitic existence. A major group of muricid parasites, the coralliophilines, lacks a radula, but these are obligate parasites, whereas *V. salebrosa* is not.

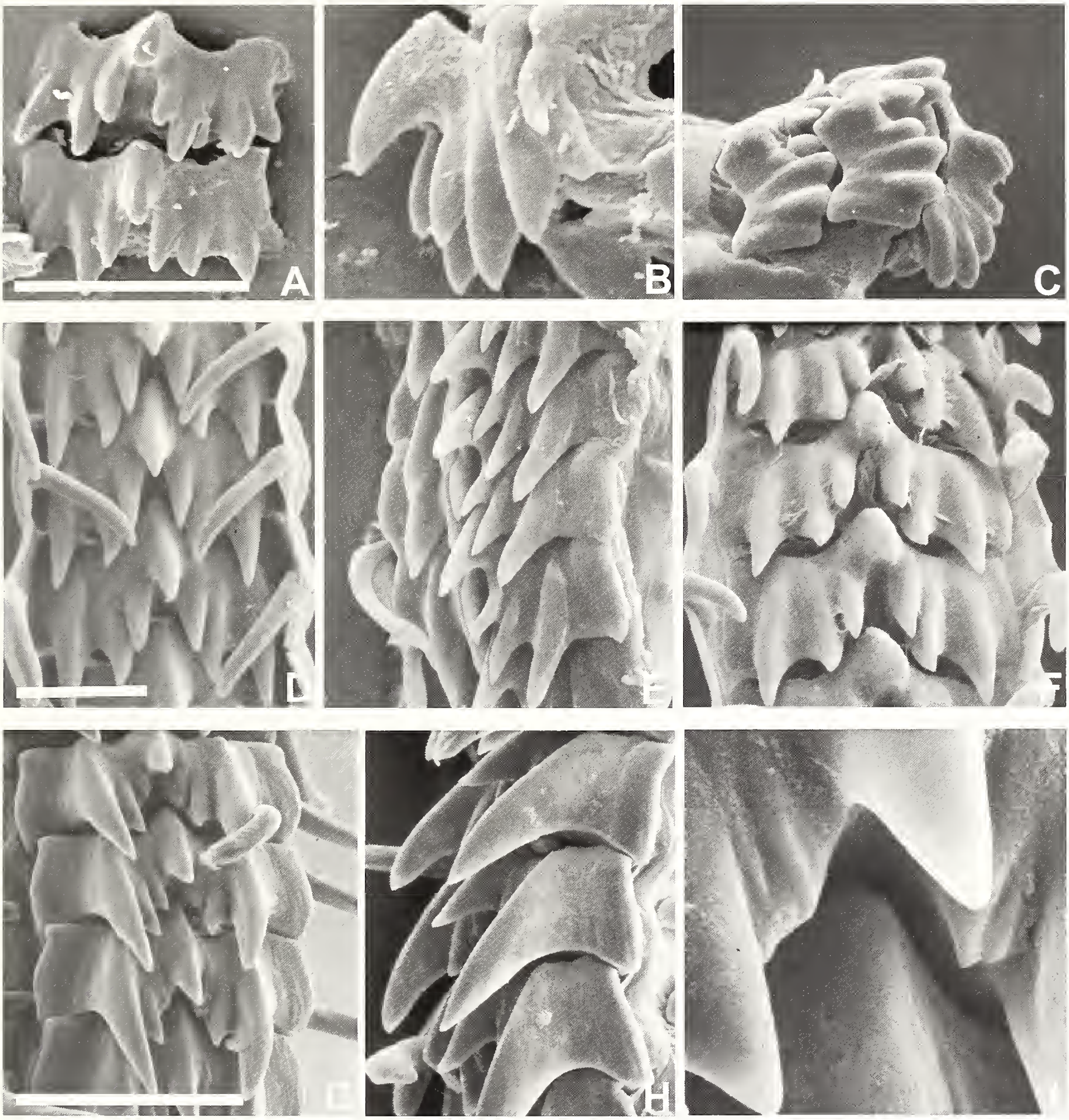

Figure 8. Radular ontogeny of the muricopsine? muricid *Vitularia salebrosa*. A-C. Front and lateral views of rachida of small juvenile with shell length of 9.9 mm, Fig. C shows worn portion of radula presumably used in feeding, locality: Venado Beach, Panama, scale bar = 5 µm. D-F. Front and lateral views of rachidia of medium-sized juvenile 31 mm in shell length, locality: Venado Beach, Panama, scale bar = 20 µm. G-I. Front and lateral views of rachidia of mature specimen with shell length of 50 mm, locality: Panama, scale bar = 50 µm.

We place this species tentatively in the Muricopsinae after Radwin and D'Attilio (1976), although this assignment was and remains controversial due to shell and opercular similarities of this genus to some members of the Ocenebrinae (Vokes 1986). In a cladistic analysis of the Muricidae based on morphological characters of the shell, ovocapsules, and radula (D. Merle and G. S. Herbert, unpubl. obs.), *Vitularia* is unequivocally placed outside the Muricopsinae and may prove to be a sister group of the Ocenebrinae. Further phylogenetic investigations are necessary to clarify the systematic position of this problematic genus within the Muricidae.

Subfamily MURICINAE
Chicoreus (Phyllonotus) pomum (Gmelin, 1791)
(Figs. 9A-D)

Material examined

Ten adult specimens of the muricine muricid *Chicoreus (Phyllonotus) pomum* were collected from shallow subtidal seagrass beds in December 2002 in St. Joseph's Bay, Florida, USA and transferred to aquaria, where they were monitored and fed regularly. Females deposited communal masses of egg capsules, and offspring hatched within several weeks. Several hundred individuals (1.0-1.5 mm shell length) hatched with a brief pediveliger stage of approx. 24 hours before absorbing the velum and using only the foot for locomotion. After a week, many juveniles began cannibalizing one another by drilling. These fully metamorphosed juveniles were collected then and preserved in 75% ethanol. Five adults (60-70 mm shell length), including males and females, were also preserved in ethanol after being relaxed in a 7.5% isotonic solution of magnesium chloride.

Ontogeny

Early post-metamorphic juveniles (1.0-1.5 mm shell length) have a rachidian that is 15 μm wide and has only three cusps of similar lengths and lying in the same plane, with intermediate denticles usually absent (Figs. 9A-B). Each rachidian of an adult snail is roughly 200 μm in width, and has a wider base, more massive cusps, a new intermediate denticle, and a more elongate central cusp (Figs. 9C-D).

Remarks

Radwin and Wells (1968) and Radwin and D'Attilio (1976) figured line drawings of the mature radula of *Chicoreus (Phyllonotus) pomum*. There are no other published illustrations of the early post-metamorphic or juvenile stage radular morphologies of this species.

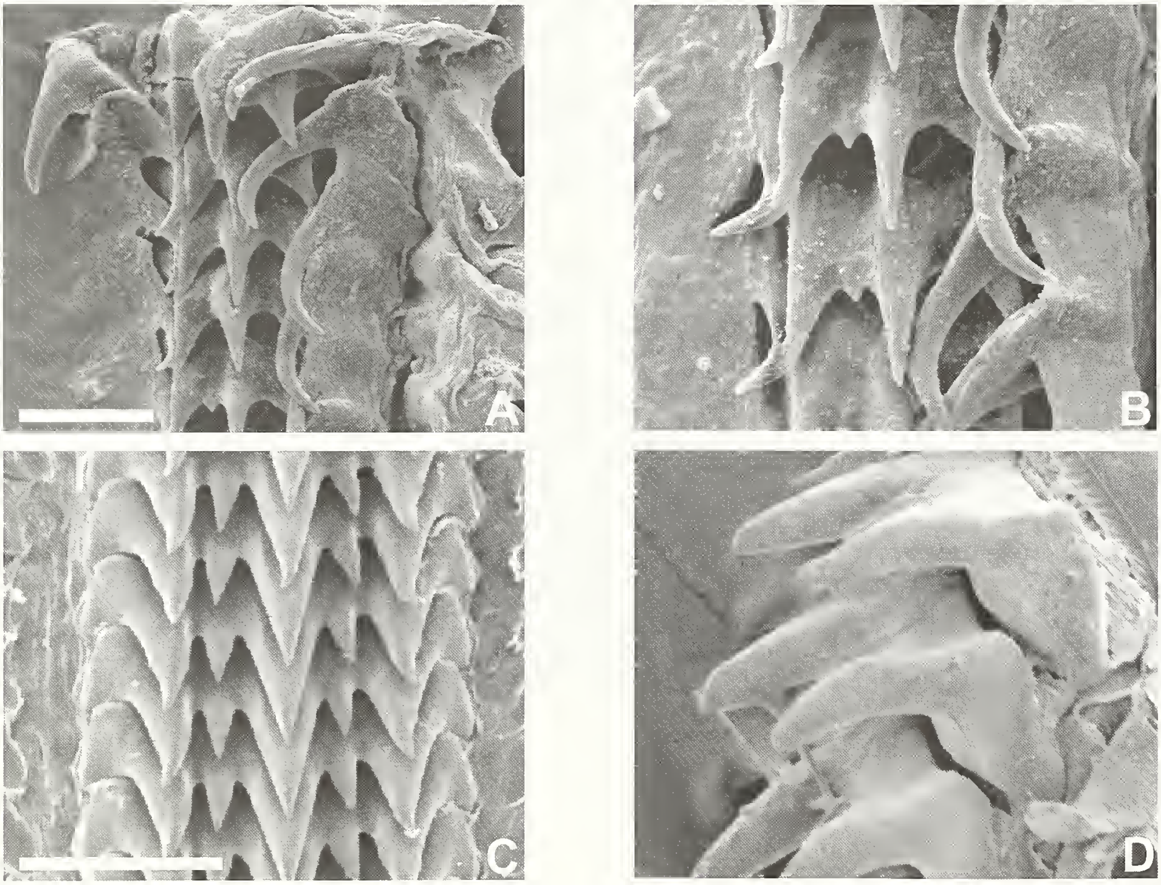

Figure 9. Radular ontogeny of the muricine *Chicoreus (Phyllonotus) pomum*. A-B. Front views of rachidia showing irregular presence of intermediate denticle in an early post-metamorphic individual with shell length of 1.5 mm, locality: St. Joseph's Bay, Florida, scale bar = 10 μm. C-D. Front and lateral views of rachidia of mature individual with shell length 63 mm, locality: St. Joseph's Bay, Florida, scale bar = 100 μm.

The absence of intermediate denticles and overall appearance of the rachidian in young individuals of *Phyllonotus pomum* gives this radular element a generalized neogastropod or "buccinoid" appearance. Similar rachidia occur in buccinids but also in olivids, melongenids, and turbinellid neogastropods, for example. Most interesting is the fact that this rachidian type has not been documented previously within the Muricidae. It is the first-known link in rachidian form between muricids and non-muricids.

DISCUSSION

Fujioka (1982, 1984a, 1984b, 1985a, 1985b) was the first to report that the muricid radula has the capacity to undergo radical transformation between subfamily-level bauplane during ontogeny. He found, specifically, that the ergalataxine monocusped rachidian likely evolved through peramorphic heterochrony, *i.e.*, extension of an ancestral rapanid ontogeny characterized by progressive reduction of all but the central cusp. With the exception of a brief treatment of the radular ontogeny of the muricine *Chicoreus (Triplex) torrefactus* by Houart (1992), however, we have, until now, known nothing of the ontogenies of the radulae of other muricids.

The present study demonstrates that major transformations between radular bauplane are nearly pervasive within the Muricidae, with transformations occurring in four of the five subfamilies studied (Fig. 10). The ontogeny of *Phyllonotus pomum* also links the seemingly disparate rachidian

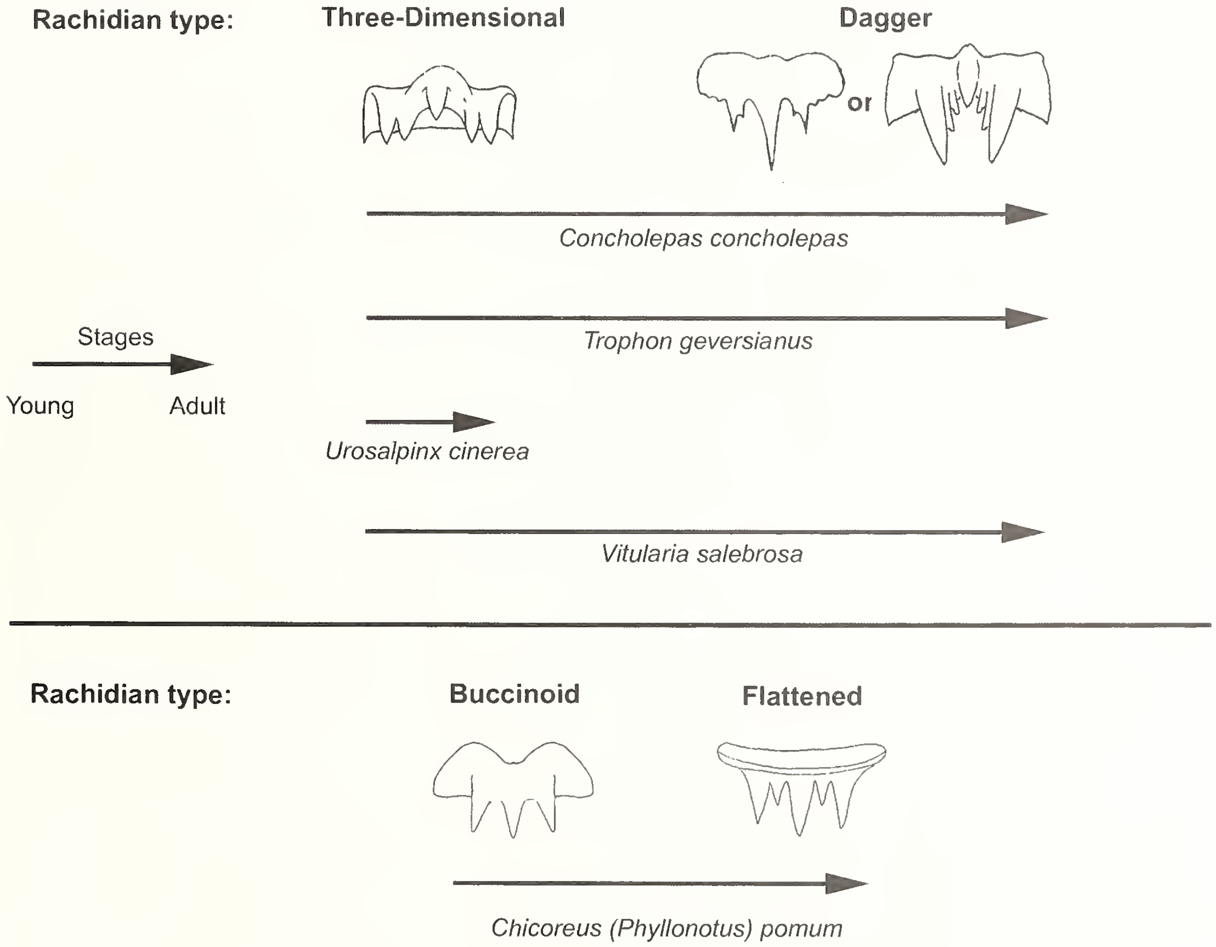

Figure 10. Generalized patterns of radular ontogeny in five species of muricid gastropod. A-B. Most of the species studied herein begin ontogeny with the three-dimensional rachidian tooth as found in the Ocenebrinae and Muricopsinae but end with a dagger rachidian typical of the Rapaninae and Ergalataxinae as well as some Trophoninae and the *Thais*-like Ocenebrinae (*i.e.*, *Nucella*, *Acanthina*, etc.). C-D. One species, *Phyllonotus pomum*, begins ontogeny with a generalized neogastropod ("buccinoid") rachidian and shifts to a flattened, pentacusped rachidian with intermediate denticles typical of the Muricidae.

morphologies of muricid and non-muricid neogastropods, and suggests that the primitive, pentacusped rachidian of the Muricidae evolved through extension of development. Equally interesting is the widespread occurrence of the 3-D rachidian that, until now, has been considered a defining trait of only the Muricopsinae and the Ocenebrinae. Data presented or reviewed in this paper demonstrate that the 3-D rachidian also occurs in at least some species of the Muricinae [*Chicopinnatus* and *Chicoreus (Triplex)*], Rapaninae (*Concholepas*), and Trophoninae (*Trophon*).

Feeding observations for one of the species studied, the rapanine *Concholepas concholepas*, suggest that the 3-D rachidian could be a functional specialization for scraping against hard substrata, whereas the dagger-type rachidian that occurs later in ontogeny could be specialized for non-drilling modes of prey subjugation. Individuals of *C. concholepas* drill shelled prey or rasp rock surfaces in search of algae exclusively during their early post-metamorphosis stage, when the animal has the 3-D rachidian, but shift to attacking prey by stabbing or lacerating the soft parts with the radula through natural orifices by the time they reach 10 to 15 mm in shell length, when it has the dagger-type rachidian (Castilla *et al.* 1979, Paine and Suchanek 1983, DiSalvo 1982, 1988, Dye 1991, DiSalvo and Carriker 1994).

Muricids that possess a 3-D rachidian as adults, *i.e.*, most species of the Ocenebrinae and Muricopsinae, also use drilling as their primary or exclusive mode of attack (G. S. Herbert, pers. obs.), again tying this rachidian bauplan to a specific drilling function. Ocenebrine and muricopsine muricids also tend to be exceedingly small relative to other taxa in the Muricidae and, thus, most similar ecologically to juvenile rather than large adult individuals of *Concholepas concholepas*. It may well be that young or small muricids lack the power to incapacitate prey using faster techniques, such as toxins or the brute force of chipping and prying (Herbert 2004), thus requiring slower methods for feeding such as drilling (Dietl and Herbert 2005) and, hence, a specialized radular type. Studies using computer modeling of the various radular morphologies within the Muricidae will be necessary to understand the exact functional basis of these radular types.

It is striking that essentially the same ontogenetic trend toward a more flattened rachidian base and elongation of just one or two cusps occurs in species that differ at all stages in important details of cusp number, position, and shape. Such differences suggest that basic structural similarities (*i.e.*, 3-D vs. flattened vs. dagger forms) among adult rachidia may be the result of independent innovation. Once the first 3-D-to-dagger ontogenetic trajectory evolved, any descendent lineages possessing this generalized ontogeny would have had the opportunity to retain the 3-D rachidian into adulthood independently through evolutionary truncation or a slowing of development. For these reasons, it is important to revisit past systematic assignments based on the bauplane that are the focus of the present study (see background section).

The repeated evolution of new traits, or innovations, has been a central theme of the muricid radiation (Vermeij 1998, 2001, Marko and Vermeij 1999, Vermeij and Carlson 2000), perhaps more so than in any other neogastropod clade. Although this phenomenon has been examined in the past from the standpoint of extrinsic factors, such as environmental conditions (*e.g.*, temperature, productivity) and community dynamics (*e.g.*, presence of incumbents, competition intensity), the ontogenetic data presented herein point to a complementary process, namely the evolution of developmental timing. Major morphological transformations during ontogeny increase the amount of phenotypic variation in the population upon which natural selection can act and, thus, the intrinsic capacity of a species to create new characters or transform existing ones. They can also reduce genetic constraints on repetitive innovation by allowing morphologies or structures already present at one stage of development to shift to later (or earlier) stages through small-scale changes in the rate or timing of development.

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Phylogenetic relationships of the columbellid taxa *Cotonopsis* and *Cosmioconcha* (Neogastropoda: Buccinoidea: Columbellidae)*

Helena Fortunato[1]

Smithsonian Tropical Research Institute, Center for Tropical Paleoecology and Anthropology, P.O. Box 0843-00153,
Balboa, Panama RP, fortunae@si.edu

Abstract: Phylogenetic reconstructions are still lacking for many molluscan groups, making evolutionary inferences much weaker. The genera *Cotonopsis* Olsson, 1942 and *Cosmioconcha* Dall, 1913 are part of the so called *Strombina* group, and as such have been used as models to study patterns of speciation and extinction brought about by the rise of the Central American gateway. Earlier work, based on a few species of each genus, pointed towards a very close relationship of these genera, which prompted a complete cladistic analysis, including all species of both genera to evaluate the level of relationship. Cladistic analyses based on shell morphology support the monophyly of the group composed by *Cotonopsis* + *Cosmioconcha*. *Cotonopsis* as currently defined is paraphyletic and includes *Cosmioconcha*. *Cotonopsis* (*Turrina*) keeps its constituency and may retain its subgeneric status. *Cotonopsis* sensu stricto should be redefined to include part of *Cosmioconcha*. *Cosmioconcha* should be subdivided into two groups. One of these groups should be included in *Cotonopsis* sensu stricto. The second group should be given subgeneric status. *Cotonopsis* has a much earlier time of origination and most probably derives from *Cosmioconcha*. Obtained results give support to some of the evolutionary patterns documented earlier for the Neogene molluscan faunas of tropical America and contribute to a better understanding of the Plio-Pleistocene divergence and turnover events related to the rise of the Panamanian land bridge.

Key words: gastropods, phylogeny, columbellids, morphology

The family Columbellidae is one of the most diverse and abundant shallow-water gastropod groups. The family has undergone rapid radiation, with over 400 species having evolved since the Danian Paleocene (Keen 1971, Abbott 1974, Radwin 1977, Tracey *et al.* 1993). The *Strombina* group sensu Jung, 1989, is one of the best known columbellid taxa, as it has been used as a model system to study evolutionary trends in species composition, diversity, and ecological patterns related to the Neogene rise of the Panama land bridge (Vermeij 1978, Jackson *et al.* 1993, 1996, Fortunato 1998, 1999). Despite these studies, only recently have the phylogenetic relationships of these taxa been investigated (deMaintenon 1994, 1999, 2005, Fortunato and Jung 1995, Fortunato 1998). *Cotonopsis* Olsson, 1942, and *Cosmioconcha* (Dall, 1913) are among the genus-level taxa that belong to this group. They are abundant and include mostly recent species with a predominantly tropical American distribution.

In his latest revision, Jung (1989) included *Cotonopsis* but excluded *Cosmioconcha* from the *Strombina* group. Work on the anatomy as well as preliminary cladistic analyses based on a subset of taxa (Fortunato and Jung 1995) confirmed Radwin's (1977) hypothesis of a possible relationship between *Cosmioconcha* and *Strombina* Mörch, 1852 based on radular and shell morphology. These results pointed to a very close relationship between *Cotonopsis* and *Cosmioconcha*. The objective of this paper it is to investigate the phylogenetic relationships of these genera, including all fossil and living species, based on shell morphology in order to better understand their history and evolution.

MATERIALS AND METHODS

This analysis includes all known fossil and Recent species of the genera *Cotonopsis* and *Cosmioconcha* (Table 1). *Cotonopsis* is a very young genus (Jung 1989), with the first known species dating from the early Pliocene of Ecuador. Most of the diversity within *Cotonopsis* developed during the Plio-Pleistocene turnover, around the time of formation of the Panama land barrier. *Cotonopsis* includes 18 species grouped in two subgenera. Only one species is known exclusively as a fossil. Of the 17 living species, 13 inhabit the eastern Pacific basin (Jung 1989), two were reported from the Caribbean region (Houbrick 1983, Petuch 1988, Fortunato 2002b), one was described from the west coast of Africa (Emerson 1993), and a fourth species was found in the Andaman Sea (Kosuge *et al.* 1998, Kronenberg and Dekker 1998, 1999).

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[1] Current address: Institut für Geowissenchaften Universitat Kiel, Ludwig-Meyn-Strasse 10, D-24118 Kiel, Germany.

Table 1. Taxa included in the phylogenetic analyses. Stratigraphic and geographic ranges are given only for ingroup taxa. LM, late Miocene; EP, early Pliocene; LP, late Pliocene. See Table 2 for more information on species of the genus *Cosmioconcha*. Extinct species indicated by an (*).

Species	Status	Stratigraphic range	Geographic range
Nassarius luteostoma (Broderip & Sowerby, 1829)	Outgroup		
Nassarius antillarum Orbigny, 1842	Outgroup		
Cantharus ringens (Reeve, 1846)	Outgroup		
Latirus concentricus (Reeve, 1847)	Outgroup		
Cotonopsis (*Cotonopsis*) *panacostaricensis* (Olsson, 1942)	Type of species of *Cotonopsis* Olsson, 1942	LP—Recent	Eastern Pacific (Costa Rica—Colombia)
Cotonopsis (*Cotonopsis*) *edentula* (Dall, 1908)		Recent	Eastern Pacific (G. of California—Panama)
Cotonopsis (*Cotonopsis*) *argentea* (Houbrick, 1983)		Recent	Caribbean (Dominican Republic)
Cotonopsis (*Cotonopsis*) *crassiparva* (Jung, 1989)		Recent	Eastern Pacific (Galapagos Is.)
Cotonopsis (*Cotonopsis*) *deroyae* (Emerson & D'Áttilio, 1969)		Recent	Eastern Pacific (Galapagos Is.)
Cotonopsis (*Cotonopsis*) aff. *deroyae* (Emerson & D'Áttilio, 1969)		Recent	Eastern Pacific (Peru)
Cotonopsis (*Cotonopsis*) *esmeraldensis* (Olsson, 1964)*		EP	Eastern Pacific (Ecuador)
Cotonopsis (*Cotonopsis*) *jaliscana* (Jung, 1989)		Recent	Eastern Pacific (Mexico)
Cotonopsis (*Cotonopsis*) *mendozana* (Shasky, 1970)		Recent	Eastern Pacific (Mexico—El Salvador)
Cotonopsis (*Cotonopsis*) *skoglundae* (Jung, 1989)		Recent	Eastern Pacific (Gulf of California)
Cotonopsis (*Cotonopsis*) *suteri* (Jung, 1989)		Recent	Eastern Pacific (Gulf of California—Mexico)
Cotonopsis (*Cotonopsis*) aff. *suteri* (Jung, 1989)		Recent	Eastern Pacific (Mexico—Costa Rica)
Cotonopsis (*Cotonopsis*) *phuketensis* (Kosuge, Roussy & Muangman, 1998)		Recent	Andaman Sea (Phuket Is.)
Cotonopsis (*Cotonopsis*) *lindae* (Petuch, 1988)		Recent	Caribbean (Barbados)
Cotonopsis (*Cotonopsis*) *monfilsi* Emerson, 1993		Recent	Western Africa (Senegal)
Cotonopsis (*Turrina*) *hirundo* (Gaskoin, 1852)		Pleistocene—Recent	Eastern Pacific (Gulf of California—Ecuador)
Cotonopsis (*Turrina*) *radwini* (Jung, 1989)		Recent	Eastern Pacific (Mexico—Panama)
Cotonopsis (*Turrina*) *turrita* (G. B. Sowerby I, 1832)		Recent	Eastern Pacific (El Salvador—Colombia)
Cosmioconcha modesta (Powys, 1835)	Type species of *Cosmioconcha* Dall, 1913	Recent	Eastern Pacific (El Salvador—Ecuador)
Cosmioconcha palmeri (Dall, 1913)		LM—Recent	Eastern Pacific (Gulf of California—Panama)
Cosmioconcha parvula (Dall, 1913)		Recent	Eastern Pacific (Gulf of California—Panama)
Cosmioconcha rehderi (Hertlein & Strong, 1951)		Recent	Eastern Pacific (Mexico—Ecuador)
Cosmioconcha pergracilis (Dall, 1913)		Recent	Eastern Pacific (Mexico)
Cosmioconcha nitens (C. B. Adams, 1850)		Recent	Caribbean (Cuba, Puerto Rico)
Cosmioconcha calliglypta (Dall & Simpson, 1901)		Recent	Caribbean (Florida, Texas, Puerto Rico)

The earliest known *Cosmioconcha* species dates from the middle Miocene. *Cosmioconcha* was first described as a subgenus of *Amphissa* H. & A. Adams, 1853 (Dall, 1913). Radwin (1978) elevated it to generic rank. *Cosmioconcha* includes seven described species, two inhabiting the Caribbean Sea and five the eastern Pacific region (Table 2, Figs. 1-2). Recent patterns of diversity and abundance of this taxon indicate a radiation similar to other paciphile genera.

Outgroup taxa were selected from Nassariidae, Buccinidae, and Fasciolariidae. Four common taxa from three buccinoidean families were selected as outgroups: Nassariidae—*Nassarius luteostoma* (Broderip & Sowerby, 1829) and *N. antillarum* d'Orbigny, 1842; Buccinidae—*Cantharus ringens* (Reeve, 1846); Fasciolariidae—*Latirus concentricus* (Reeve, 1847). These taxa were selected based on availability and not on the presumption of close phylogenetic relationship.

Forty-two qualitative characters were identified (Appendix 1). Shell sculpture is one of the most characteristic elements of this group, and provides numerous diagnostic characters. Fourteen characters code for type and sculptural details of the teleoconch and body whorl. Presence of shoulder, constriction, inflation, and angulation of the whorls, as well as presence and strength of humps were also coded. Apertural elements (thickness, denticles, apertural and columellar calluses and plicae, parietal ridge) used in traditional taxonomy of this group of gastropods are included here as well. Other characters are general shell shape, type of spire, type and depth of suture, and the relation between the total height and the height of the body whorl. All taxa were coded from direct observation.

MacClade 3.0 (Maddison and Maddison 1992) was used to create the data matrix of 25 taxa and 42 morphological characters (Appendix 2). The heuristic search in PAUP 4.0b10 (Swofford 2001) was used for the analyses, using a random addition sequence with ten replicate searches performed. All characters were unordered and weighted equally. Clade support was assessed through a bootstrap procedure (100 bootstrap replicates with 10 random addition sequences). Tree support was determined using Bremer decay analysis (Bremer 1994) in which progressively longer trees are saved and their consensus calculated in order to see how many more steps are required to collapse branches.

RESULTS

Cladistic analyses of the data matrix in Appendix 2 yielded six most-parsimonious trees (L=218 steps, CI=0.303, RI=0.513, and RC=0.155). Only the strict consensus tree (Fig. 3) is presented here (the 50% majority rule consensus tree shows exactly the same topology).

The ingroup is monophyletic in all trees, consisting of a single clade grouping all *Cotonopsis* and *Cosmioconcha* species. This clade is defined by fusiform shells with high spire and mostly un-sculptured earlier teleoconch whorls, body whorl mostly un-sculptured, apertures with moderately thickened outer lips, and a well developed, recurved anterior canal.

The genus *Cotonopsis*, as traditionally constructed, is paraphyletic and includes the polyphyletic *Cosmioconcha*. The subgenus *Cotonopsis* (*Turrina*) emerges as a monophyletic crown group. *Cosmioconcha* species are grouped in two separate clades within *Cotonopsis*. One clade, which contains the type species of *Cosmioconcha*, emerges in an unresolved trichotomy with a small clade containing the type species of *Cotonopsis* and a large clade that includes *Cotonopsis*, the remaining *Cosmioconcha*, and *Cotonopsis* (*Turrina*).

All species assigned to *Cotonopsis* sensu stricto emerge as a grade that also includes a small clade of three species of *Cosmioconcha*, including its type species. These species have mostly stout shells with axially sculptured late spire whorls and well defined cords at the base of the body whorl. They have broad apertures with denticles and thin outer lip edges.

This grade (all *Cotonopsis* sensu stricto + three *Cosmioconcha* taxa) has several smaller groupings. Its base is weakly resolved with several *Cotonopsis* species branching succes-

Table 2. Synopsis of species belonging to the genus *Cosmioconcha* Dall, 1913. *, type species.

Genus	Species	Author & Year	Synonyms
Cosmioconcha	*modesta**	Powys, 1835	*Buccinum modestum* Powys, 1835; *Strombina laevistriata* Li, 1930
	palmeri	Dall, 1913	
	parvula	Dall, 1913	
	rehderi	Hertlein & Strong, 1951	
	pergracilis	Dall, 1913	
	nitens	C. B. Adams, 1850	*Fusus nitens* C. B. Adams, 1850; *Columbella* (*Astyris*) *perpicta* Dall & Simpson, 1901; *Mitrella perpicta* (Dall & Simpson) Woodring, 1928
	calliglypta	Dall & Simpson, 1901	*Anachis calliglypta* Dall & Simpson, 1901

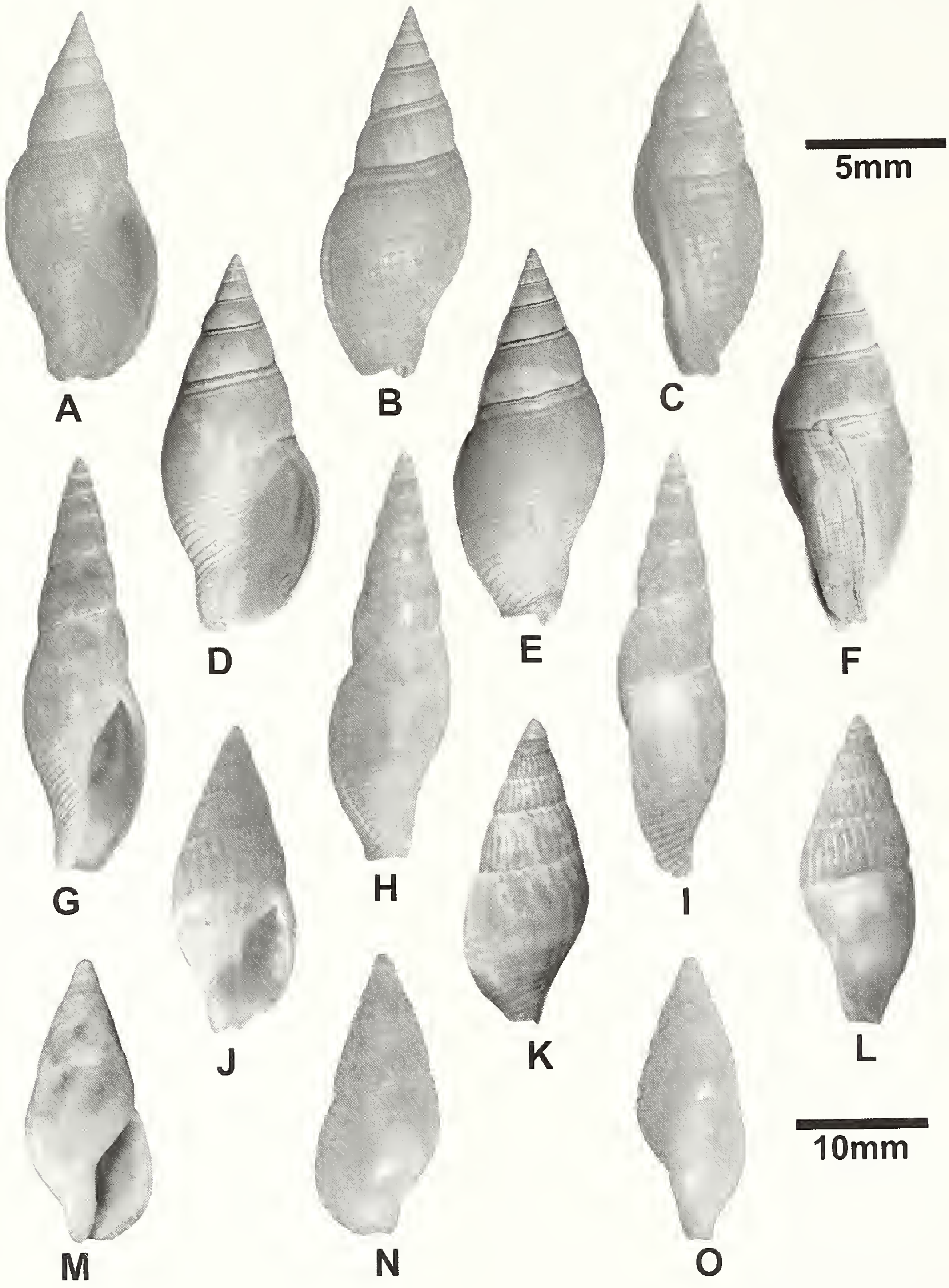

Figure 1. Species of *Cosmioconcha* Dall, 1913. A-C *Cosmioconcha modesta* (NMB 17442). D-F *Cosmioconcha palmeri* (NMB 17793). G-I *Cosmioconcha parvula* (NMB H18181). J-L *Cosmioconcha rehderi* (NMB H18182). M-O *Cosmioconcha nitens* (NMB 18567). Views are front, rear, and from right side. All specimens belong to the Gibson-Smith Recent collection housed at the Naturhistorisches Museum Basel, Switzerland.

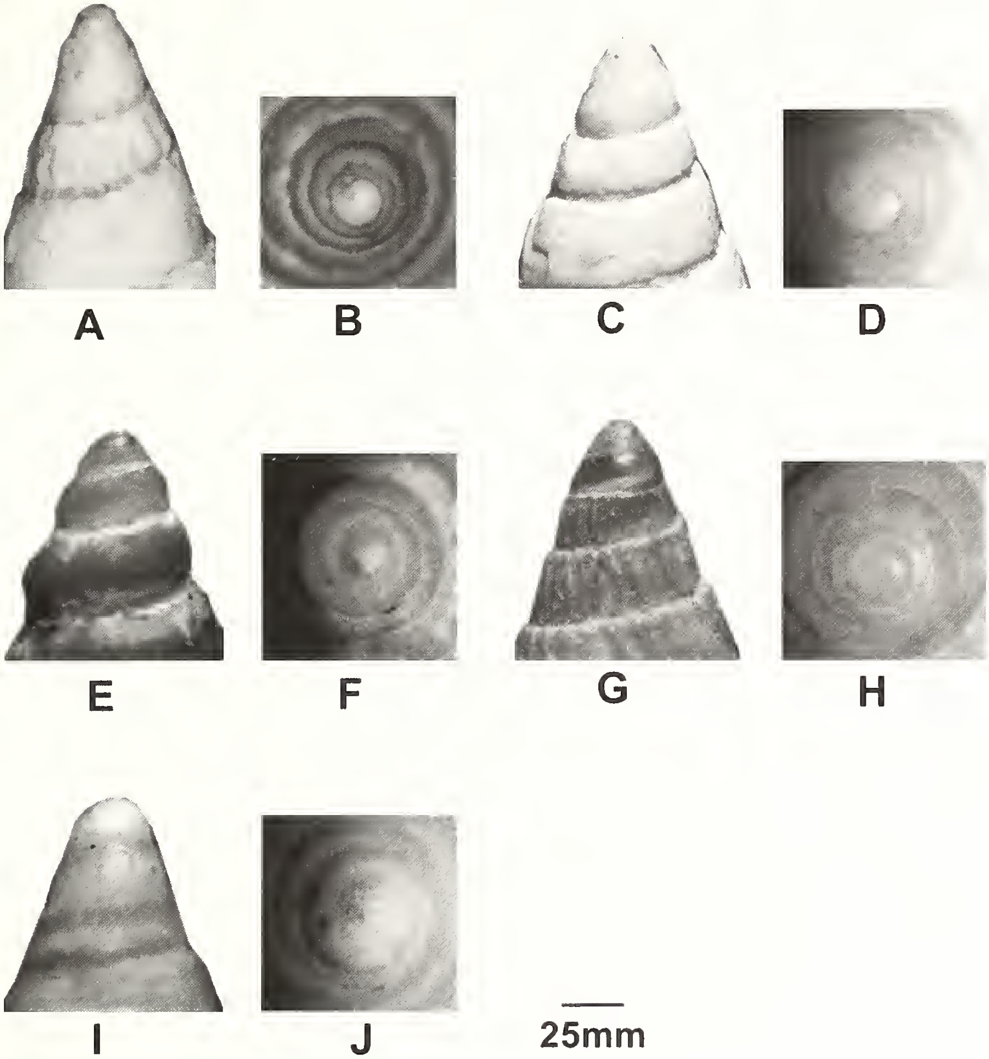

Figure 2. Species of *Cosmioconcha* Dall, 1913, protoconchs. A-B *Cosmioconcha modesta.* C-D *Cosmioconcha palmeri.* E-F *Cosmioconcha parvula.* G-H *Cosmioconcha rehderi.* I-J *Cosmioconcha nitens.* Same specimens as in Fig. 1.

sively. Among these are *C. monfilsi* and *C. lindae*, an African and a Caribbean species respectively. The next branch has two small subclades, one with three *Cosmioconcha* and a second one joining two *Cotonopsis* taxa. Next to diverge is the *Cotonopsis* living in the Andaman Sea, followed by another small group formed by two eastern Pacific *Cotonopsis*. The last grouping of this grade joins a Caribbean and two eastern Pacific *Cotonopsis* taxa.

The two other groups are sister clades and are located as crown groups. One of these clades groups, but does not resolve, the three *Cotonopsis* (*Turrina*) taxa. The second group is composed by four *Cosmioconcha* species, among which appear the two Caribbean species. Species of the crown clades have slender shells, almost no axial ornamentation on the spire whorls, and narrower apertures with a thicker edge. The species of *Cosmioconcha* have more convex spires, numerous denticles on the aperture, and a collar like band below the spire suture. *Cotonopsis* (*Turrina*) taxa are characterized by taller, straight-sided shells and a well developed thickening behind the outer lip. Results of the Bremer decay analysis are plotted onto the strict consensus tree (Fig. 3).

The first round resulted in 284 trees with 219 steps or less. These trees support the monophyly of the entire ingroup as well as the monophyly of both crown clades, *i.e.* *Cotonopsis* (*Turrina*) and the four *Cosmioconcha* species. Two small groups that appear among the basal branches (one joining three eastern Pacific *Cosmioconcha*, and another with two *Cotonopsis* sensu stricto taxa) are equally supported here. The second round of the decay yielded 6,278 trees, 220

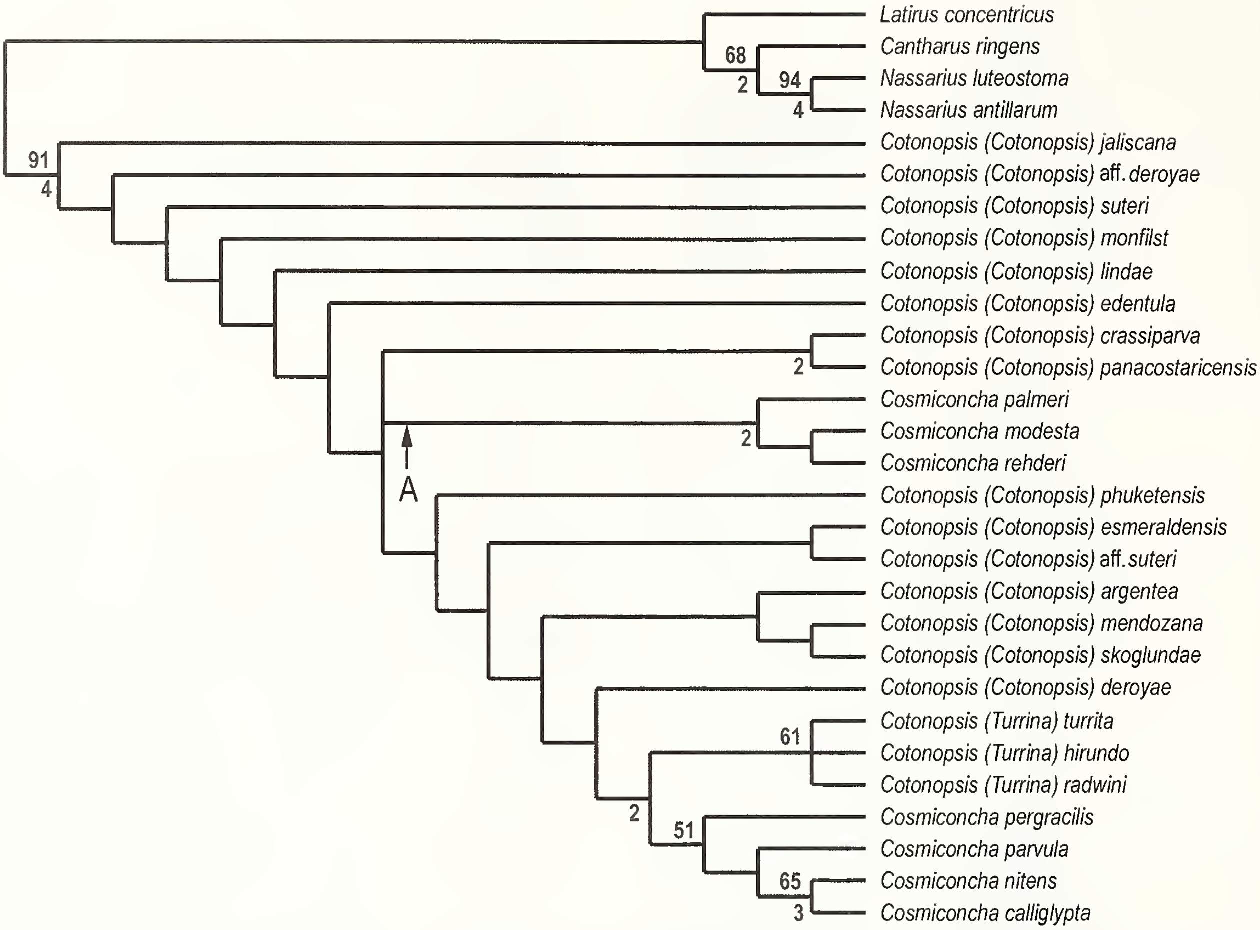
Latirus concentricus
Cantharus ringens
Nassarius luteostoma
Nassarius antillarum
Cotonopsis (Cotonopsis) jaliscana
Cotonopsis (Cotonopsis) aff. deroyae
Cotonopsis (Cotonopsis) suteri
Cotonopsis (Cotonopsis) monfilst
Cotonopsis (Cotonopsis) lindae
Cotonopsis (Cotonopsis) edentula
Cotonopsis (Cotonopsis) crassiparva
Cotonopsis (Cotonopsis) panacostaricensis
Cosmiconcha palmeri
Cosmiconcha modesta
Cosmiconcha rehderi
Cotonopsis (Cotonopsis) phuketensis
Cotonopsis (Cotonopsis) esmeraldensis
Cotonopsis (Cotonopsis) aff. suteri
Cotonopsis (Cotonopsis) argentea
Cotonopsis (Cotonopsis) mendozana
Cotonopsis (Cotonopsis) skoglundae
Cotonopsis (Cotonopsis) deroyae
Cotonopsis (Turrina) turrita
Cotonopsis (Turrina) hirundo
Cotonopsis (Turrina) radwini
Cosmiconcha pergracilis
Cosmiconcha parvula
Cosmiconcha nitens
Cosmiconcha calliglypta
68
2
94
4
91
4
2
2
A
61
2
51
65
3

Figure 3. Strict consensus tree of the six most parsimonious cladograms. Numbers below branch nodes are Bremer support values, *i.e.*, the number of extra steps necessary to collapse that node. Nodes without values collapse with one extra step. Numbers above branch nodes are bootstrap support values for that node.

steps or less. The ingroup is still monophyletic but only the group with the two Caribbean *Cosmioconcha* species is supported. The third round yielded 117,655 trees, 221 steps or less. It supports the monophyly of the ingroup but there is no resolution. The fourth round of decay analyses overflowed the memory with over 500,000 trees. The monophyly of the ingroup is still supported here. The bootstrap support for some of the clades is plotted in the strict consensus tree (Fig. 3).

DISCUSSION

The main objective of this work was to re-evaluate all species that have traditionally been assigned to the genera *Cotonopsis* and *Cosmioconcha,* in order to assess their relationships and the true constituency of these genera. Earlier analyses based on a selected subset of species from each of these genera (Fortunato and Jung 1995) suggested a close relationship of these taxa, confirming Radwin's (1977) hypothesis of a relationship between *Cosmioconcha* and the *Strombina* group of which *Cotonopsis* is part (Jung 1989). Our objective was to test this relationship, including in the analysis all known species currently included in both genera.

The results of this study indicate that *Cotonopsis* + *Cosmioconcha* form a monophyletic group. *Cotonopsis* as it was initially defined by Jung (1989) is paraphyletic and contains *Cosmioconcha.* Of the two subgenera of *Cotonopsis,* only *Cotonopsis* (*Turrina*) is monophyletic and retains its entire constituency. *Cotonopsis* sensu stricto, as currently constructed, is paraphyletic. Its status as a monophyletic taxon could be restored only by synonymizing both *Cosmioconcha* and *Turrina.* Alternatively, inclusion of more closely related outgroups might alter the rooting of the tree. Rooting at Position A (Fig. 3) would be required to retain monophyletic *Cotonopsis* and *Cosmioconcha* as sister taxa although the majority of species currently assigned to *Cosmioconcha* would still emerge *Cotonopsis.*

Species assigned to *Cosmioconcha* are divided into two groups. The first group is composed of three eastern Pacific species, and is located near the base of the tree, among *Cotonopsis* sensu stricto taxa. This group includes the type species, *Cosmioconcha modesta*. The second group is one of the crown subclades and unites two Caribbean and two eastern Pacific species. This group is sister of *Cotonopsis* (*Turrina*).

Within the grade *Cotonopsis* sensu stricto, *C. monfilsi*, a deep water species from West Africa, and *C. lindae*, a shallow water species from Barbados, form adjacent branches but are flanked by eastern Pacific species from California, Mexico, and Peru that have no known fossil record. It is tempting to speculate about the possible existence of geminate pairs [*i.e.*, closely related taxa separated by a barrier (Jordan 1908)] among the extinct fossil ancestors of these taxa. These relationships also suggest an earlier radiation of American species, probably from the eastern Pacific towards the Atlantic before the closure of the Panamanian Strait. Unfortunately, none of these species have a known fossil record which could help calibrate the time of such radiation. However, both the geographic distribution of these species, and the fact that several of the following taxa (within the context of this tree topology) have fossils dating back to the middle Miocene (*i.e.*, *Cosmioconcha palmeri* (Dall, 1913)) suggest that such a radiation may have taken place during the middle Miocene. It is also reasonable to assume the possible existence of fossil lineages yet to be found. Molecular studies could provide an alternative tool to elucidate these relationships.

Three eastern Pacific species of *Cosmioconcha*, including the type species, emerge as a clade. The stem species, *C. palmeri*, has the oldest fossil record of all the species in this study, being known from the middle Miocene deposits of Darien [Radwin, 1977; Panama Paleontological Project (PPP) data]. Based on these results, it is reasonable to assume that *Cotonopsis* sensu stricto is much older than postulated by Jung (1989) in his revision of the *Strombina* group. Jung indicated an early Pliocene age for *Cotonopsis*, based on the occurrence of *Cotonopsis esmeraldensis* (Olsson, 1964) in the early Pliocene of Ecuador. Results of the present analysis indicate that *Cosmioconcha* is part of *Cotonopsis* sensu stricto, thus moving the time of origination of this genus most probably to middle Miocene.

Another closely related small clade unites the Recent *Cotonopsis* (*Cotonopsis*) *crassiparva* and a late Pliocene *Cotonopsis* (*Cotonopsis*) *panacostaricensis* (Olsson, 1942), the type species of *Cotonopsis*.

Cotonopsis (*Cotonopsis*) *phuketensis* (Kosuge *et al.*, 1998), a shallow water species from the Andaman Sea, is the second species in this genus with a distribution outside of tropical America. There are infrequent reports of planktotrophic larvae crossing the central Pacific barrier (Scheltema, 1978). Most *Cotonopsis* have planktotrophic larvae (exceptions are *C. jaliscana*, *C. esmeraldensis*, and *C. argentea*; authorities in Table 1) able to spend a considerable amount of time in the plankton (Fortunato 2002a). Again, the lack of fossil data precludes the dating of this dispersal event. Nevertheless, the presence of an early Pliocene species within a sister clade indicates that it may date back to the early Pliocene, at the very least. Here again, molecular data would be useful to help resolve these events.

The next clade comprises *Cotonopsis esmeraldensis* (early Pliocene of Ecuador) and a recent eastern Pacific species, *C.* aff. *suteri*. This is probably a case of speciation with a switch in developmental mode, as *C. esmeraldensis* is a non-planktotroph whereas its sister species has planktonic larvae. *Cotonopsis esmeraldensis* is the only extinct taxon in the analyzed data set. The basal species of this clade, *C. pukhetensis*, is also a planktotroph. A trans-isthmian event in the history of the group is documented in the next branch of this phylogenetic tree. *Cotonopsis argentea*, a non planktotroph taxon found in deep water of the Dominican Republic coast is the sister taxon of two eastern Pacific species (*C. mendozana* and *C. skoglundae*).

The crown of the tree is composed by two subclades with a relatively strong Bremer and bootstrap support. The stem taxon is *Cotonopsis deroyae*. One of the groups includes the three *Cotonopsis* (*Turrina*) species, confirming the composition and monophyly of this subgenus. The second group, composed by four *Cosmioconcha* species, documents another trans-isthmian event: *C. nitens* and *C. calliglypta* are shallow water taxa inhabiting the Caribbean Sea that diverged from an eastern Pacific taxon.

Based on the obtained results and the phylogenetic reconstruction presented here, *Cotonopsis* sensu stricto, as presently understood, represents a grade that includes several *Cosmioconcha* taxa (*i.e.*, *C. palmeri*, *C. modesta*, and *C. rehderi*), among them the type species of *Cosmioconcha*. All have stout shells with high spires, axially sculptured early teleoconch whorls, body whorls with strong cords on the base, and wide apertures.

The four "*Cosmioconcha*" species that constitute one of the crown groups of the tree are not closely related to the type species of *Cosmioconcha*. These species are characterized by smaller fusiform shells, absence of sculpture on the early teleoconch, absent or weak cords on the body whorl, and narrow apertures. The character "presence of a collar-like band below the suture", traditionally used to unify *Cosmioconcha* taxa is not reliable and should not be given more value than any other morphological character.

Cotonopsis is a taxon that reflects the pulse of origination that occurred in the eastern Pacific at the Pleio-Pliocene boundary. Most of the recognized taxa originated during the last two million years, probably along the shallow waters of the eastern Pacific coast. Unfortunately, the stratigraphic record of the eastern Pacific region is not very well preserved (Coates *et al.* 1992, Jackson *et al.* 1993, 1996) and there is no fossil record for most of the known species. *Cosmioconcha* also originated in this region and has a fossil record that dates back to the middle Miocene. Based on the phylogenetic reconstruction presented here it is reasonable to assume that *Cotonopsis* derives from a *Cosmioconcha*-like ancestor. The group then radiates and speciates with the documented increase in species diversity towards the recent, a pattern well documented for the entire *Strombina* group (Jung 1989, Jackson *et al.* 1993, 1996).

The *Strombina* group has been used as a model system to document patterns of diversification during the Neogene rise of the Panamanian isthmus. Phylogenetic inferences have started to give historical support to earlier studies. The taxa studied here are part of this group and the results confirm the validity of the evolutionary patterns documented earlier (Jackson *et al.* 1993, 1996, Fortunato and Jung 1995, Fortunato 1998, 1999). It is also reasonable to assume the existence of fossil lineages and even Recent taxa yet to be found that could contribute to a better understanding of the natural history of the molluscan fauna of the region and its relationships.

AKNOWLEDGMENTS

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Appendix 1. Character and character state list.

1–Shell shape: (0) fusiform (elongate, spire high); (1) strombiform, spire low; (2) buccinoid (stout, spire tapering); (3) columbelloid (stout, spire high)
2–Shape of spire whorls: (0) straight sided; (1) straight going to convex; (2) straight going to concave
3–Depth of suture: (0) shallow; (1) impressed; (2) incised
4–Shoulder on spire whorls: (0) absent; (1) present, inconspicuous; (2) present, strong
5–Number of whorls in protoconch: (0) <2; (1) 2-3; (2) >3
6–Axial sculpture on early teleoconch whorls: (0) absent; (1) present, inconspicuous and subordinate; (2) present, well developed
7–Spiral sculpture on early teleoconch whorls: (0) absent; (1) present, inconspicuous and subordinate; (2) present, well developed
8–Axial sculpture on late spire whorls: (0) absent; (1) present, inconspicuous and subordinate; (2) present, well developed
9–Spiral sculpture on late spire whorls: (0) absent; (1) present, inconspicuous and subordinate; (2) present, well developed
10–Spiral sculpture on body whorl: (0) absent; (1) present, inconspicuous and subordinate; (2) present, well developed
11–Axial sculpture on body whorl: (0) absent; (1) present, inconspicuous and subordinate; (2) present, well developed
12–Shoulder on body whorl: (0) absent; (1) present
13–Cords on base of body whorl: (0) absent; (1) present, weak; (2) present, well developed
14–Concavity on central part of body whorl: (0) absent; (1) present
15–Constriction on lower part of body whorl: (0) inconspicuously constricted; (1) strongly constricted
16–Inflation of body whorl: (0) not inflated; (1) inflated
17–Type of sculpture on early vs. late spire whorls: (0) same; (1) different
18–Shape of aperture: (0) broad; (1) narrow; (2) slit-like
19–Thickness of outer lip: (0) not thickened; (1) slightly thickened; (2) conspicuous thickness
20–Teeth on inner surface of outer lip: (0) absent; (1) present, small and inconspicuous; (2) present, strongly developed
21–Number of teeth on inner surface of outer lip: (0) none; (1) few (1-5); (2) numerous (>5)
22–Posterior canal: (0) absent; (1) present, inconspicuous; (2) present, well developed
23–Apertural callus: (0) absent; (1) present, as a slight thickness; (2) present, continuous, well developed
24–Columellar denticles: (0) absent; (1) present;
25–Parietal callus: (0) absent; (1) present, slightly thickened; (2) present, well developed
26–Parietal denticles: (0) absent; (1) present
27–Parietal ridge: (0) absent; (1) present, small and inconspicuous; (2) present, well developed
28–Sinus on outer lip: (0) absent; (1) present
29–Flaring of outer lip: (0) absent; (1) present
30–Length of anterior canal: (0) short; (1) intermediate; (2) long
31–Width of anterior canal: 90) wide; (1) narrow
32–Extension of adapical part of outer lip (aperture edge at suture): (0) outer lip not extended; (1) outer lip somewhat extended after suture
33–Shape of anterior canal: (0) slightly curved; (1) strongly curved; (2) straight
34–Notch of anterior canal (at the end): (0) shallow; (1) deep depression
35–Thickening behind outer lip: (0) absent; (1) present, slight thickness; (2) present, well developed
36–Dorsal hump: (0) absent; (1) present, slight thickness; (2) present, well developed
37–Edge of outer lip: (0) sharp; (1) rounded
38–Hump on left side of outer lip: (0) absent; (1) present, slight thickness; (2) present, well developed
39–Repeated thickenings behind outer lip: (0) absent; (1) present
40–Plicae on columella: (0) absent; (1) present
41–Relation aperture height/total height: (0) aperture <½ total shell height; (1) aperture much smaller than ½ total shell height; (2) aperture bigger than ½ but smaller than ¾ total shell height
42–Collar-like band below spire suture: (0) absent; (1) present

Appendix 2. Character matrix used for analyses.

Species	Characters				
Nassarius luteostoma	2111221222	2220120012	2220202010	0022101001	00
Nassarius antillarum	2111222222	2120120002	2220202010	0022101001	00
Cantharus ringens	2102021222	2220120001	2211112010	0012200000	00
Latirus concentricus	0202021222	2120110001	2010100012	2020000002	10
Cotonopsis (Cotonopsis) argentea	0100020200	1010100111	1011101002	0011100000	00
Cotonopsis (Cotonopsis) crassiparva	0120120211	0020110022	1120202011	0011100000	00
Cotonopsis (Cotonopsis) deroye	0100120011	0010100121	1120102002	1011100000	00
Cotonopsis (Cotonopsis) edentula	0120120000	0020111010	0110101012	0111100000	00
Cotonopsis (Cotonopsis) jaliscana	0121020000	0110111121	2120102010	0000100000	00
Cotonopsis (Cotonopsis) mendozana	0100120000	0010111021	1020201010	0011100000	00
Cotonopsis (Cotonopsis) panacostaricensis	0120120211	1020110011	2120102012	0011100000	00
Cotonopsis (Cotonopsis) skoglundae	0100?22021	0010001012	2020201002	1011100000	00
Cotonopsis (Cotonopsis) suteri	0111210000	0120111021	1120121002	0010100000	00
Cotonopsis (Cotonopsis) esmeraldensis	0100020111	0120111122	1020101002	0111101000	10
Cotonopsis (Cotonopsis) aff. *deroye*	0101?10100	1100111011	2110102000	0000101000	10
Cotonopsis (Cotonopsis) aff. *suteri*	0110120111	0120101011	1220001001	0010100000	00
Cotonopsis (Cotonopsis) phuketensis	0120110010	0020101111	2020100001	0001100000	00
Cotonopsis (Cotonopsis) lindae	0100110100	0020111012	2211102001	0101100000	00
Cotonopsis (Cotonopsis) monfilsi	0121212011	0120100022	2111101000	1121101000	00
Cotonopsis (Turrina) turrita	0000100010	0010001120	0210102002	0001200100	00
Cotonopsis (Turrina) hirundo	0000100010	0010101020	0210001012	1011200000	00
Cotonopsis (Turrina) radwini	0000100010	0010111020	1210102010	0011200000	00
Cosmioconcha palmeri	0220120010	0120111032	2221200011	0011100000	01
Cosmioconcha modesta	3220101011	0010001022	2220201010	0001200000	01
Cosmioconcha rehderi	3200220210	2020011031	1220201011	0121200000	11
Cosmioconcha nitens	0210100010	0010001021	2110100000	0021100000	11
Cosmioconcha calliglypta	0200110110	0010011021	2110100000	0021101000	11
Cosmioconcha parvula	0200100011	0010101022	2110100002	0001101000	01
Cosmioconcha pergracilis	0200200010	0010101021	1110100002	0001101000	01

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Family Pseudolividae (Caenogastropoda, Muricoidea): A polyphyletic taxon*

Luiz Ricardo L. Simone

Museu de Zoologia da Universidade de São Paulo, Cx. Postal 42494, 04299-970 São Paulo, SP, Brazil, lrsimone@usp.br

Abstract: A detailed morphological study was performed on the following taxa normally considered to belong to the family Pseudolividae: (1) *Zemira australis* (Sowerby, 1833) from Australia; (2) *Fulmentum ancilla* (Hanley, 1859) from South Africa; and (3) *Melapium lineatum* (Lamarck, 1822) from South Africa. Two additional species of pseudolivids, *Benthobia atafona* Simone, 2003 and *B. complexirhyna* Simone, 2003, from Brazil and New Zealand respectively, are considered. Two other muricoideans are included in this study: (1) *Nassodonta dorri* (Watteblet, 1886) [Nassariidae] from Vietnam (morphological study also included) and (2) *Siratus senegalensis* (Gmelin, 1791) (Muricidae) from Brazil (published elsewhere). Both species are outgroups, but operationally included as part of the ingroup in order to test the monophyly of the Pseudolividae. In particular, *N. dorri* has a shell very similar to a pseudolivid. A complete taxonomical and morphological treatment of each species is included, as a scenario of a formal phylogenetic analysis. Additional outgroups considered include a pool of Tonnoidea (the root) and Conoidea. The cladogram is: (Tonnoidea (Conoidea ((*Benthobia atafona–B. complexirhyna*) (*Nassodonta dorri* (*Zemira australis* (*Fulmentum ancilla* (*Siratus senegalensis–Melapium lineatum*))))))). Analyses of each important character and of the cladogram were performed. Some of the conclusions include that the family Pseudolividae, as presently understood, is polyphyletic, as it would include a nassariid (*N. dorri*) and a muricid (*S. senegalensis*).

Key words: Neogastropoda, polyphyly, morphology, phylogeny

The taxon Pseudolividae Fisher, 1884, had been previously used by several researchers (*e.g.*, Cossmann 1901, Golikov and Starobogatov 1975, Squires 1989), but it was better defined as a family by Kantor (1991), based on anatomical features of basal neogastropods. The family reunites genera previously considered as belonging to several other families, including, *e.g.*, Cancellariidae (*Benthobia* Dall, 1889), Buccinidae (*Buccinorbis* Conrad, 1865), and Olividae (*Melapium* Adams and Adams, 1853; *Pseudoliva* Swainson, 1840; *Sylvanocochlis* Melvill, 1903; *Zemira* Adams and Adams, 1853). This taxonomy was followed by some researchers (*e.g.*, Vermeij and DeVries 1997, Bouchet and Vermeij 1998, Pacaud and Schnetler 1999, Nielsen and Frassinetti 2003). Moreover, Vermeij (1997, 1998) revised the family Pseudolividae, including fossil species, establishing its origin in the late Cretaceous. A more complete history of the concept of the family can also be found in that paper. However, some authors still considered the family as a subtaxon of Olividae (*e.g.*, Hayes 1994, Smith 1998) (Pseudolivinae). Although our knowledge of pseudolivid species is relatively rich, particularly with regard to anatomy (*e.g.*, Ponder and Darragh 1975, Kantor 1991, Simone 2003), the definition of the family remains unclear, and no phylogenetic analysis has yet been performed, other than that of Kantor (1991).

The main difficulty in studying pseudolivids is finding preserved animals. Pseudolivids are normally rare and found in deep waters, which precludes obtaining a large set of samples for an extensive anatomical study. Although the pseudolivids are more abundant as fossils, with about a hundred species (Vermeij 1998), they are relatively poor in diversity in the Recent fauna, with about 10-15 living species. As about a third of the species are available for study, belonging to the different branches of the family, a study of them appears to be worthwhile, at least in terms of testing the monophyly of the group and identifying anatomical characters that would better define it.

This paper is part of a larger project on the phylogenetic definition of the Caenogastropoda based on detailed morphology, this time focusing the Pseudolividae. One of the genera, *Benthobia* Dall, 1889, was published elsewhere (Simone 2003), and the species of remaining genera are included herein.

Nassodonta dorri (Watteblet, 1886), from Vietnam, one of the few freshwater neogastropods known, belonging to the family Nassariidae, has a shell similar in morphology to those of pseudolivids (Kantor and Kilburn 2001). This taxon is also included in this study to test the monophyly of the Pseudolividae, as the shell characters certainly can converge.

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* From the symposium "Relationships of the Neogastropoda" presented at the meeting of the American Malacological Society, held 31 July-4 August 2004 at Sanibel Island, Florida.

MATERIALS AND METHODS

Most specimens used in this study belong to institutional collections. The specimens were dissected by standard techniques, under a microscope, with the specimens immersed in water. Some organs such as the oviduct and foregut were processed by standard histological techniques to obtain serial sections 5 µm in thickness and stained using Mallory trichrome. Hard structures, such as shells, radulae, and jaws were also examined using the SEM in the "Laboratório de Microscopia Eletrônica do Museu de Zoologia da Universidade de São Paulo". The descriptive part of this paper provides a complete description of the first species; the remaining species are described under a comparative aspect, with most of similar features omitted. This measure is adopted for decreasing the length of this paper and for optimizing the data. The same approach is adopted in the figures. A detailed list of examined species follows each species description.

The section on comparative morphology is organized as a phylogenetic analysis. The account on each character begins with abbreviated descriptive sentence followed by plesiomorphic and derived conditions(s); also included are CI and RI (consistency and retention indices, respectively) and values for the character under the most parsimonious hypothesis. Following the apomorphic state(s), a list of terminal taxa with the apomorphic condition is presented. Hundreds of characters were selected, based on the examined samples. Those that emerged as autapomorphic, highly variable, or overlapping, were selected but not included in the cladistic analysis. The remaining characters were organized into states, coded, polarized comparing with outgroups, and a cladistic analysis was performed.

Other previously studied Caenogastropoda were selected as outgroups. They are mainly the following: Cerithioidea (Simone 2001), Stromboidea (Simone 2005), Cypraeoidea (Simone 2004a), Calyptraeoidea (Simone 2002), and architaenioglossans (Simone 2004b). In the discussion, some specific outgroup taxa are mentioned, based upon observed or published data. However, in the matrix of characters (Table 1) only 4 taxa are shown, the ground plan of the Conoidea and Tonnoidea. The ground plan of these superfamilies are chosen in the sense of being representative; however, the final result is the same if the ground plan were substituted by any one of the 64 (terminal) species present in those papers. Two additional species are included, *Siratus senegalensis* (Gmelin, 1790) [Muricidae], based on unpublished observations from another, ongoing study, and the nassariid *Nassodonta dorri*, mentioned above. Each character, state, and polarization is justified in the discussion section which includes a concise explanation when warranted.

The discussion of each character is also based on the analysis of the resulting tree (Figs. 1-2) although the matrix of characters (Table 1) and the subsequent tree (Figs. 1-2) are shown only in the following section.

The synapomorphies of the ingroup (superfamily autapomorphies) are preserved in the present paper, because they are the main concern as referred to in the introduction. The ingroup autapomorphies are the basis for a better establishment of a still imprecise taxon. They confirm the internal position of some possible "outgroups" such as tonnoideans and conoideans. They will be useful in the ongoing phylogenetic study of the entire order Caenogastropoda as the ground plan of the superfamily. Additionally, they are in agreement with some phylogenetic approaches used in studies of other groups (*e.g.*, Yeates 1992, Pinna 1996).

Some multistate characters are here analyzed under an additive (ordered) approach. In each case, the additive concept is justified in the discussion and is always based on the ontogeny, or because each state represents a clear modification of the preceding one. Additionally, each additive multistate character was also analyzed as non-additive, and any fortuitous change in the result and/or indices are also reported. The cladistic analysis was performed with the aid of the computer program "Tree Gardner 2.2" (Ramos 1997), which basically works as an interface of the Hennig86 (Farris 1988). The used algorithm was "ie". The computer program PAUP was also used, mainly to determine the robustness of the nodes. Both programs presented the same result.

Table 1. Matrix of characters of ingroup and two outgroups (bottom).

		1		2		3	
Taxon/character	12345	67890	12345	67890	12345	67890	1234
Benthobia atafona	10101	00121	10001	00011	21010	20101	1111
Benthobia complexirhyna	10101	00121	10001	00011	21010	20101	1111
Zemira australis	11010	01120	01111	10011	01121	11101	0111
Melapium lineatum	00100	13010	01111	11111	11121	21010	0011
Fulmentum ancilla	11110	00010	01101	11011	21111	11110	1111
Nassodonta dorri	10110	01011	00111	10111	?1110	2??01	0?11
Siratus senegalensis	00010	12000	01111	11111	11111	01110	0211
Conoidea	00100	00000	00001	00001	00010	00001	0011
Tonnoidea	00000	00000	00000	00000	00000	00000	0000

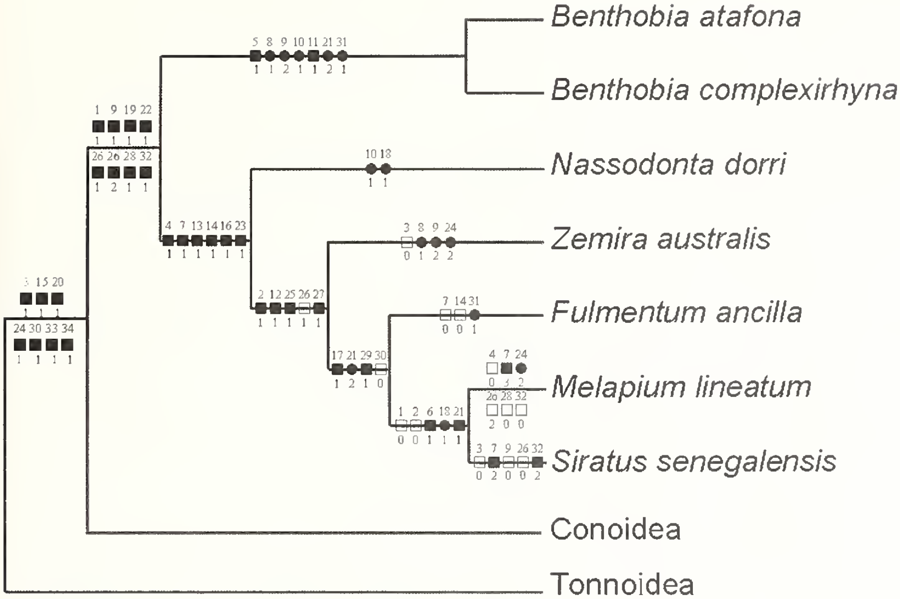
Benthobia atafona
5 8 9 10 11 21 31
1 1 2 1 1 2 1
Benthobia complexirhyna
1 9 19 22
1 1 1 1
26 26 28 32
1 2 1 1
10 18
1 1
Nassodonta dorri
4 7 13 14 16 23
1 1 1 1 1 1
3 8 9 24
0 1 2 2
Zemira australis
3 15 20
1 1 1
24 30 33 34
1 1 1 1
2 12 25 26 27
1 1 1 1 1
7 14 31
0 0 1
Fulmentum ancilla
17 21 29 30
1 2 1 0
4 7 24
0 3 2
Melapium lineatum
26 28 32
2 0 0
1 2 6 18 21
0 0 1 1 1
3 7 9 26 32
0 2 0 0 2
Siratus senegalensis
Conoidea
Tonnoidea

Figure 1. Single most parsimonious tree based on the data matrix in Table 1, with three outgroups operationally analyzed as part of the ingroup (Conoidea, *Siratus*, and *Nassodonta*). Length: 62; CI = 66; RI = 69. Each symbol indicates a synapomorphy supporting each node (only the homoplastic autapomorphies are shown) as follows: full square = non-homoplastic synapomorphy; circle = convergence; empty square = reversion.

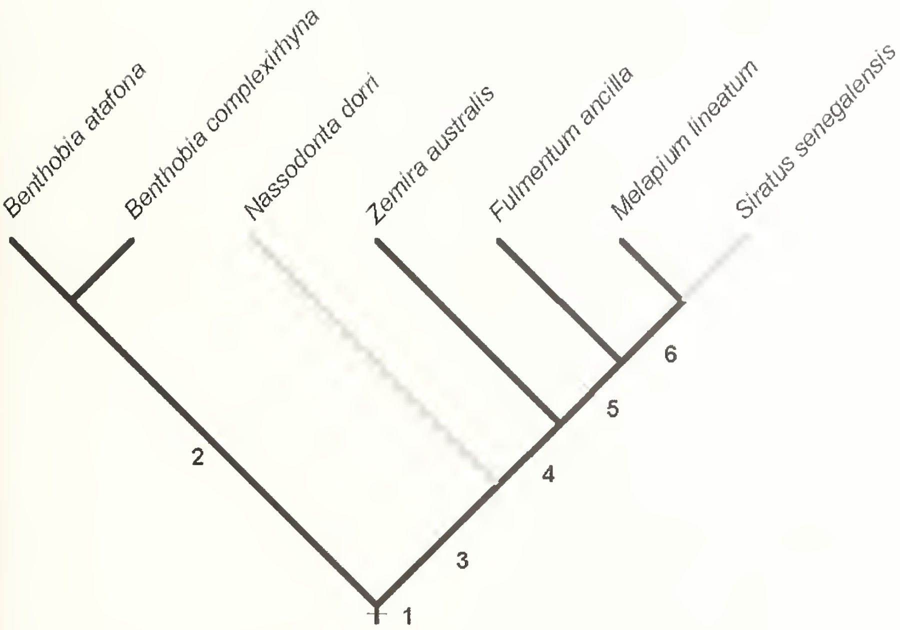
Benthobia atafona
Benthobia complexirhyna
Nassodonta dorri
Zemira australis
Fulmentum ancilla
Melapium lineatum
Siratus senegalensis
6
5
2
4
3
1

Figure 2. Single most parsimonious tree (same of Fig. 1, excluding both more basal outgroups) (Length: 62; CI = 66; RI = 69), with the nodes numbered. The gray branches represent the non-pseudolivid taxa, left branch a member of the Nassariidae, right branch a member of the Muricidae. The black branches represent the taxa mostly considered to be Pseudolividae, showing the polyphyletic nature of the taxon.

SYSTEMATICS

Genus *Zemira* Adams and Adams, 1853
(Type species *Eburna australis*, by monotypy)

Zemira australis (Sowerby, 1833)
(Figs. 3A-F, 4A-B, 5A-7H)

Synonymy: see Ponder and Darragh 1975: 101.
Complement:
Zemira australis: Ponder and Darragh 1975: 89-97, 101-104 (text figs. 1, 2, pl. 7 fig. 1-2. pl. 8 figs. 12-24); Smith 1998: 835-836 (fig. 15.165-C).

Description

Shell (Figs. 3A-C, 3F). Fusiform, pale brown, opaque. Protoconch, spherical, smooth, opaque, of about one whorl; boundary between protoconch and teleoconch unclear. Spire pointed, about half of length of body whorl. Suture well-marked by a subsutural, concave, wide groove, from protoconch up to outer lip; surface of groove smooth, external edge elevated, forming a low carina. Remaining regions sculptured by uniform, spiral, narrow furrows, about nine in penultimate whorl, about 10 in body whorl; one of these furrows, located between middle and anterior thirds of body whorl, deeper and wider (Figs. 3B-C). No umbilicus except a narrow furrow in inferior third around inner lip (Fig. 3A). Peristome oval, white, glossy (Figs. 3A, 3F). Canal short and narrow, left edge truncate, right edge wanting, as continuation of outer lip. Outer lip simple, cutting edge, rounded; very short tooth between middle and inferior thirds correspondent to deeper spiral furrow of body whorl (Figs. 3C, 3F); wide notch in superior region, at some distance from suture correspondent to sub-sutural carina. Inner lip simple, callus narrow, slightly more transparent than inner region of peristome.

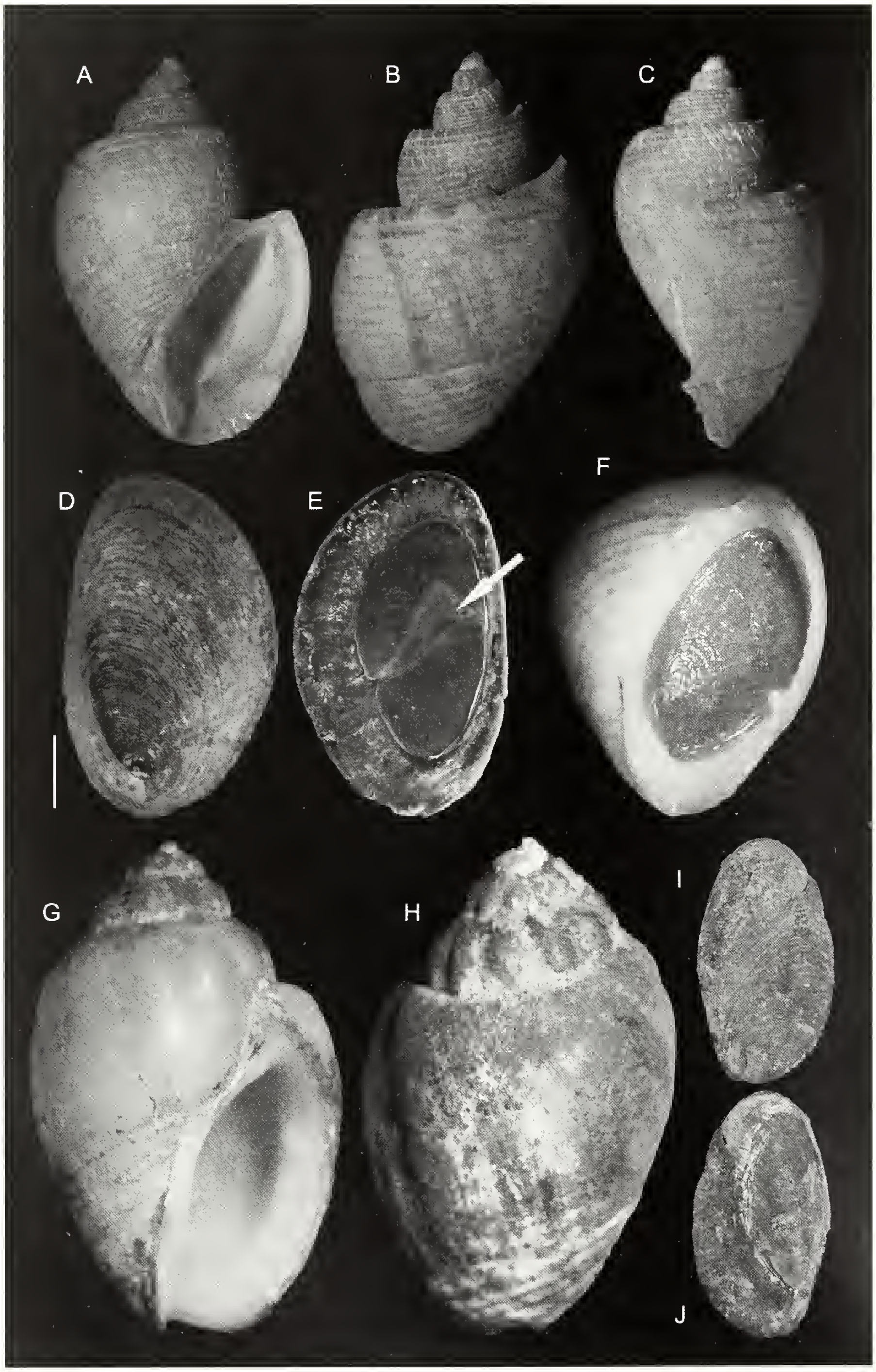

Figure 3. Shells and opercula. A-F, *Zemira australis*, AMS 333288. A-C, shell (specimen #1), female, apertural, dorsal and lateral views, length = 18.6 mm; D-E, operculum, outer and inner views, arrow indicates separation of two scar regions, scale bar = 2 mm; F, detail of aperture closed by operculum (specimen #4), showing labral tooth and opercular sculpture. G-J, *Nassodonta dorri*, MZSP 53533; G-H, shell, apertural and dorsal views, length = 13.6 mm; I-J, operculum, outer and inner views, length = 5 mm.

Head-foot (Figs. 5A, 5C, 5F). Head weakly protruded, bilobed, with single region pigmented by dark brown, the rest pale cream. Tentacles located close to each other and close to median line; tentacles' base very wide, flat, flap-like, outer edge rounded; distal half of tentacles marked by abrupt narrowing of the base, narrow, tapering gradually; tentacles' tip rounded. Foot broad, of about half whorl when retracted. Sole oval, edges thick and rounded. Anterior furrow of pedal glands deep, straight, thick superior and inferior edges, not reaching lateral-anterior end. Lateral region of the sole of the foot clearly extending beyond remaining dorsal regions of foot, division marked by a shallow longitudinal furrow lying somewhat in middle region between sole edge and dorsal region of foot (Fig. 5A). Opercular pad elliptical, almost as wide as the dorsal surface of the foot; possessing clear, median, oblique difference in levels (Fig. 5C); posterior half of this division forming small area with different, iridescent color. Columellar muscle thick, of about one half whorl. Male with large penis in posterior-right region behind the right tentacle described below.

Operculum (Figs. 3D-F). Elliptical, horny, pale to reddish brown. Nucleus sub-terminal, located closer to interior-inner edge. Outer surface with normal concentric growth lines, and series of radial lines produced by minute, aligned scales located on the growth lines, from nucleus to edges (Fig. 3F). Low carina running at some distance from inferior and inner edges, from nucleus up to middle level of inner edge. Inner surface glossy. Scar elliptical, occupying about 2/3 of inner area, somewhat dislocated closer to inner edge. Scar having two different levels of about the same area, one superior and another inferior; both separated by a wide chevron, marking a low step (Fig. 3E, arrow); a small notch in region where the chevron touches outer scar edge.

Mantle organs (Figs. 5B, 5D). Mantle edge simple, thick. Siphon small, not extending beyond mantle edge. Osphradium about 1/3 the width of the pallial cavity and ¾ of its length. Osphradium filaments tall, central region scalloped by 5 folds in the left and 6 folds in the right filaments (Fig. 5B: os). Osphradium filaments widely attached along mantle roof. Osphradium anterior end curved to the left, with left filaments clearly smaller than right filaments; remaining osphradial regions with somewhat symmetrical filaments (left filaments slightly smaller). Very narrow area between osphradium and gill. Ctenidial vein narrow, dislocated weakly beyond left gill edge, towards right edge of osphradium. Gill slightly longer than the osphradium and of about the same width; its anterior end broadly pointed, located closer to the mantle edge, far from anterior end of osphradium; posterior gill end located slightly posterior to that of osphradium. Afferent gill vessel very narrow, lying at a short distance from the right edge of the gill. Between the gill and the right edge of pallial cavity there is an area equivalent in width to that of the gill. The hypobranchial gland is thin, greenish beige, covering most of the area between the gill and the rectum, including the left and ventral surfaces of the rectum; the anterior region of the hypobranchial gland tapers gradually. Rectum narrow, running along the right edge of the pallial cavity (Figs. 5B, 5D-E). Anus simple, sessile, located between middle and anterior thirds of pallial cavity. Pallial gonoducts located between rectum and pallial floor, described below.

Visceral mass (Fig. 5D). Anterior whorl mostly occupied by stomach, kidney, and pericardium (Figs. 5E, 6E). Digestive gland greenish brown, located along inferior region of each visceral whorl, covering middle digestive tubes and also two whorls poste-

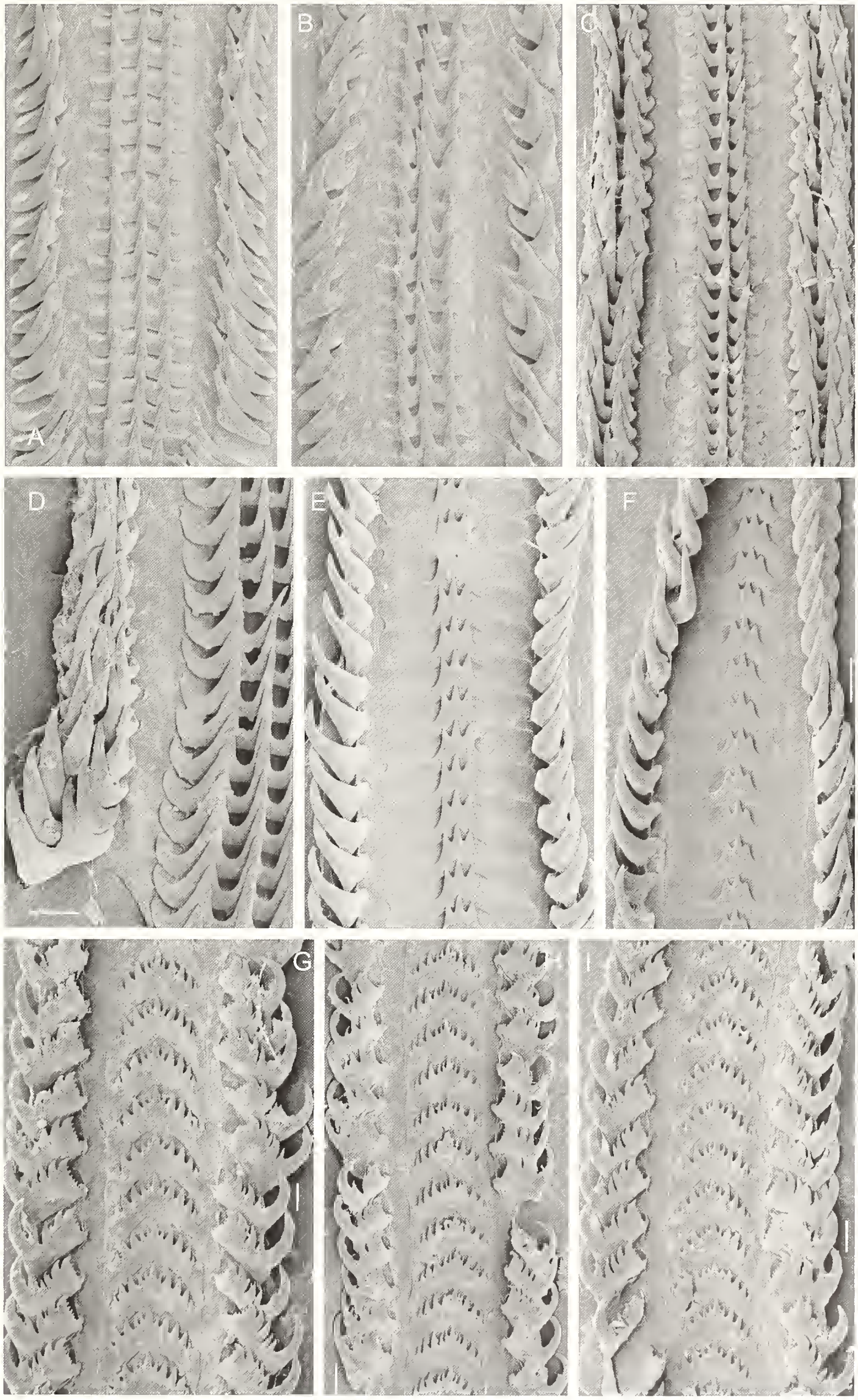

Figure 4. Scanning electron micrographs of radulae. A-B, *Zemira australis*, scale bars = 30 μm. C-D, *Fulmentum ancilla*, scale bars = 50 μm. E-F, *Melapium lineatum*, scale bars = 50 μm. G-I, *Nassodonta dorri*, scale bars = 30 μm.

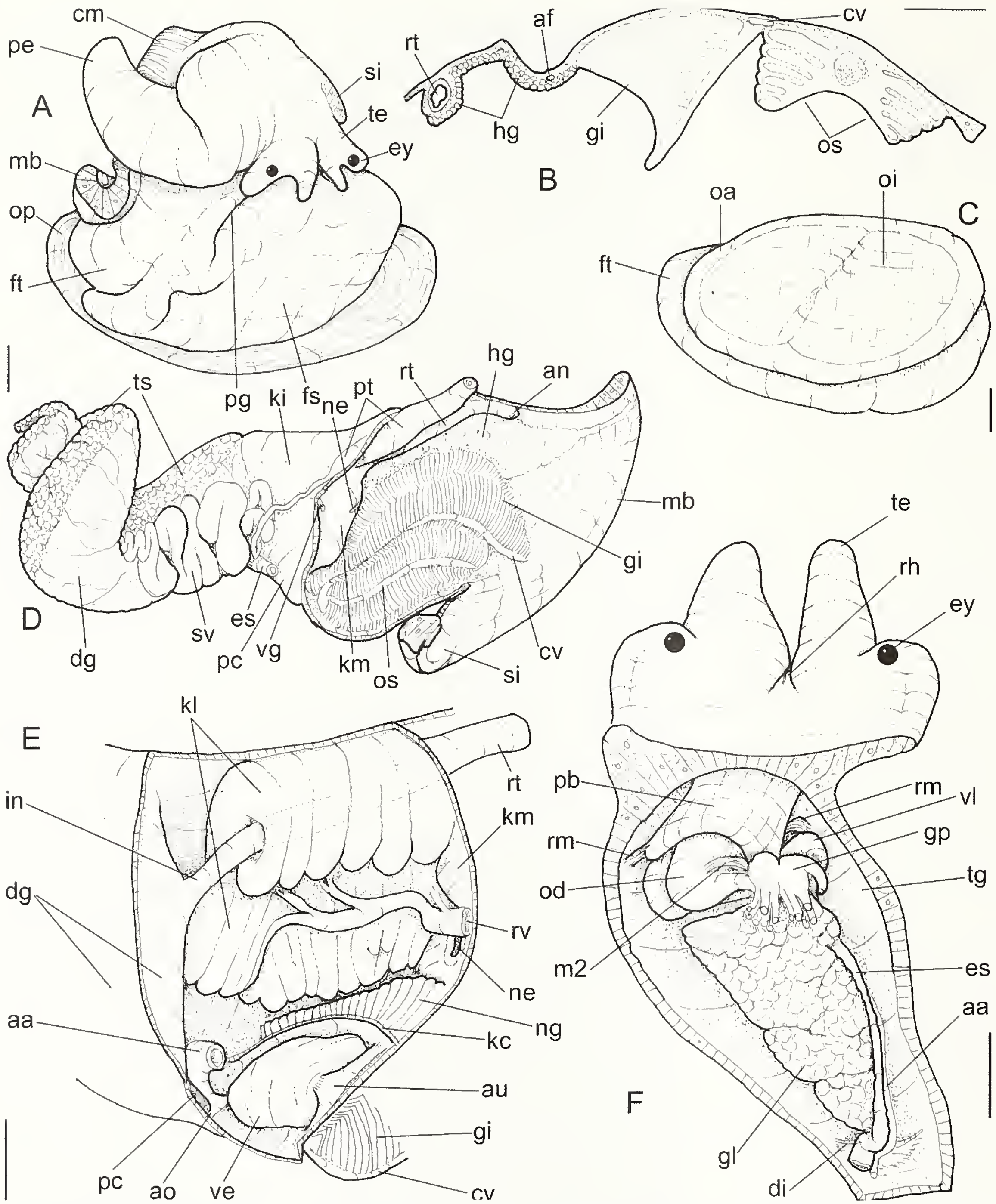
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Figure 5. *Zemira australis* anatomy. A, head-foot, male, frontal view. B, pallial cavity roof, transverse section at middle level of osphradium. C, foot, detail of opercular pad, dorsal view, operculum removed. D, pallial cavity, ventral-inner view, and visceral mass, male. E, region of kidney, ventral view, ventral wall of kidney and pericardium removed, anterior membrane partially deflected to right. F, head and haemocoel, ventral view, foot removed. Scale bars = 1 mm. Abbreviations listed in section with figure captions.

rior to stomach. Gonad pale beige, lying along superior and columellar surfaces of visceral whorls posterior to stomach.

Circulatory and excretory systems (Fig. 5E). Pericardium located just posterior to gill, along the left anterior region of the visceral mass (Fig. 5D). Auricle small, triangular, attached to anterior surface of pericardium, with the ctenidial vein entering from the left and the connection to kidney at its right end. Auricle connected to anterior surface of ventricle. Ventricle very large, filling most of pericardium volume. Aortas located along posterior region of the ventricle; anterior aorta about 4 times larger than posterior aorta, and located ventral to it. Kidney occupies about 1/3 of pallial cavity volume, located along middle and right regions of the anterior end of the visceral mass. Nephridial gland triangular in section, broader anteriorly, gradually narrowing posteriorly; lying along the dorsal region of the renopericardial wall. Renal lobe occupying most of the kidney's interior volume, presenting two flaps of similar thickness, fused along right region; ventral flap shorter (about half of dorsal flap), intestine running through it; dorsal flap occupying most of renal dorsal surface. Afferent renal vessel large, running from the haemocoel, covering right side of nephropore, with some branches inserted in inner surface of dorsal flap of renal lobe.

Digestive system (Figs. 5F-8A). **Proboscis** relatively short (about 1/3 of haemocoel length) (Figs. 5F, 7A: pb). Mouth transverse along proboscis tip. Buccal cavity with pair of broad and tall lateral folds, each one dividing within a short distance, one branch running to the odontophore tube, the other to the esophagus (Fig. 5F). Ventral surface between buccal folds with a clear, low, flat, chitinous platform (Fig. 7F: ol). Odontophore oval, about half the length of the proboscis (Figs. 7A, 7E). Odontophore tube connecting it with buccal cavity. **Odontophore** muscles (Figs. 6A-D, 7E-F): **m1**, several small muscle fibers connecting buccal mass to adjacent inner surface of proboscis; **mj**, pair of peribuccal muscles and protractor of odontophore, origin thin within dorsal wall of oral cavity, running along odontophore tube becoming thicker, inserting into outer surface of cartilages, externally to m6 and medially to m4, in two branches, one anterior and another posterior, posterior branch about twice the size of and longer than the anterior branch; **m2**, pair of retractor muscles of odontophore, originating in ventral surface of haemocoel, in region just posterior to proboscis (when retracted), running dorsally, with median fibers running through nerve ring, inserting into posterior surface of odontophore, part into m5 and part into m4 regions close to median line; **m2a**, auxiliary of m2, being single and running between both m2, attached to ventral surface of anterior aorta; its fibers apparently originated ventral to nerve ring, not passing through it (Figs. 6A-D); **m3**, pair of thin dorsal protractor muscles of odontophore, originating in anterior-dorsal end of odontophore tube, at its juncture with the esophagus, running posteriorly, covering dorsal surface of odontophore tube, inserting into odontophore middle-dorsal surface (Figs. 7E-F); **m4**, strong pair of dorsal tensor radular muscles, originating in odontophore cartilages along a line surrounding their ventral surface, running towards dorsal surrounding lateral surface of cartilages, inserting laterally along radular sac into the region near the buccal cavity; **m5**, pair of secondary dorsal tensor muscles of radula, originating in posterior and medial regions of the cartilages, running dorsal and medial as continuation from m4, inserting into radular sac near the buccal cavity alongside and medial to m4 insertion; **m6**, thin horizontal muscle, uniting both odontophore cartilages, with about 3/4 of cartilage length, inserting along a line into the ventral and external surfaces of the cartilages, at a short distance from their inner-ventral edge, starting at the anterior end of the cartilages and ending just before their posterior quarter (Figs. 6B-C); **m11a**, pair of ventral tensor muscles of radula, thin, somewhat broad, originating partly in the posterior-ventral end of cartilages and partly in the m2 insertion, running anteriorly covering m6, inserting into ventral edge of radula and subradular cartilage, and some inner portion preceding this (Fig. 6D); **m14**, pair of ventral protractor muscles of odontophore, originating along ventral surface of oral tube and tube of odontophore, running posteriorly at short distance from median line, covering central surface of odontophore, inserting into posterior-ventral surface of odontophore, close to m2 insertion (Fig. 7E). Other non-muscular odontophore structures: **sc**, subradular cartilage, expanding in exposed region of radula into buccal cavity, covering neighboring surface of radula (Figs. 6A, 6D, 7F); **oc**, odontophore cartilages, somewhat elliptical, flat, with medial-ventral edge slightly straighter than outer edge, posterior region clearly narrow (Figs. 6B, 6C); **br**, subradular membrane, covering inner surface of subradular cartilage and radula, m4, m5, and m11a insertions. **Radula** (Figs. 4A-B): **rachidian** tooth with short transverse base, spanning about 1/3 of radular ribbon, 3 long, tall (about 2/3 of base length), sharp pointed cusps somewhat equidistant from each other, central cusp symmetrical, outer cusps weakly turned outwardly; between rachidian and lateral teeth a distance equivalent to 1/3 of rachidian width; **lateral tooth** hook-like, base broad (equivalent to 2/3 of rachidian base width), gradually narrowing up to sharply pointed tip, height about 1.5 that of rachidian; straight to weakly curved inwardly. **Salivary glands** clustering along anterior region of valve of Leiblein and ventral ganglia of nerve ring, attaching to lateral surface of the anterior esophagus just anterior to the valve of Leiblein (Figs. 7A-B); their ducts very narrow, totally at-

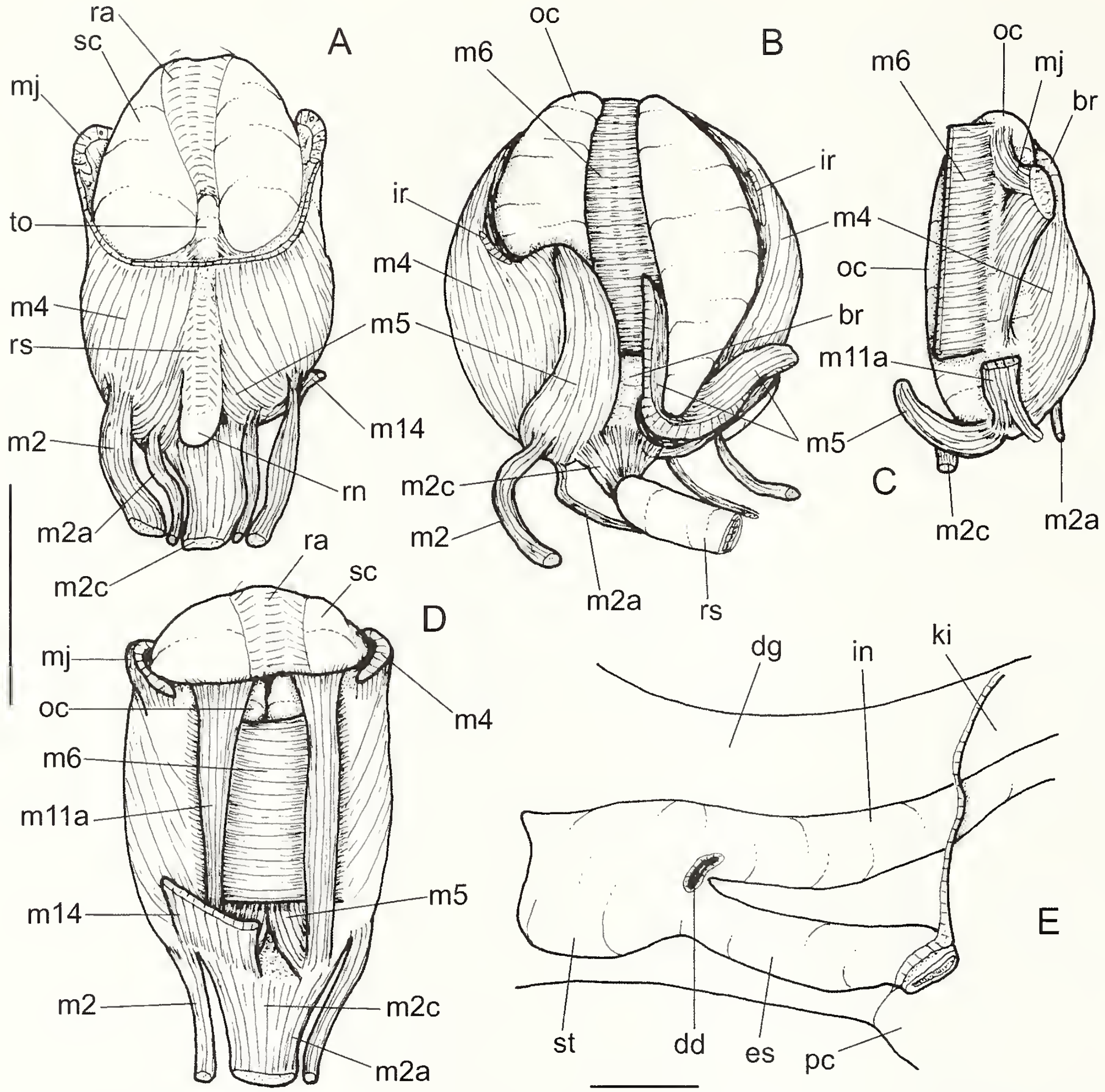

Figure 6. *Zemira australis* anatomy. A, odontophore, dorsal view, anterior odontophore tube removed. B, same, outer layer of muscles and radular apparatus removed and only partially shown (rs). C, odontophore, ventral view, only its right side shown, posterior muscles deflected. D, same, outer view, outer layer of muscles and membrane partially removed. E, midgut, ventral view as *in situ*, some adjacent structures also shown. Scale bars = 1 mm. Abbreviations listed in section with figure captions.

tached to anterior esophagus wall and to lateral wall of oral tube; opening very small (Fig. 7F: sa), into anterior region of lateral folds of buccal cavity, somewhat ventrally, just within the anterior end of a narrow furrow surrounding the ventral edge of the odontophore tube folds. **Anterior esophagus** with somewhat thick walls, length equivalent to that of odontophore, inner surface with lateral, longitudinal, low, and flat folds that become narrower posteriorly. Between these folds are secondary, low, narrow folds (Fig. 7F). **Valve of Leiblein** with about 1/4 of odontophore volume, anterior region with a transverse, white band into which long cilia insert, middle and posterior regions pale beige, corresponding to a tall inner gland occupying most of inner surface; oblique furrow (by pass) present, separating all valve regions (Figs. 7A-B); inner surface smooth, not glandular, bordered by pair of low and very narrow folds that diverge in its anterior region, and continuous with middle esophagus folds posteriorly. **Middle esophagus** about half as long as

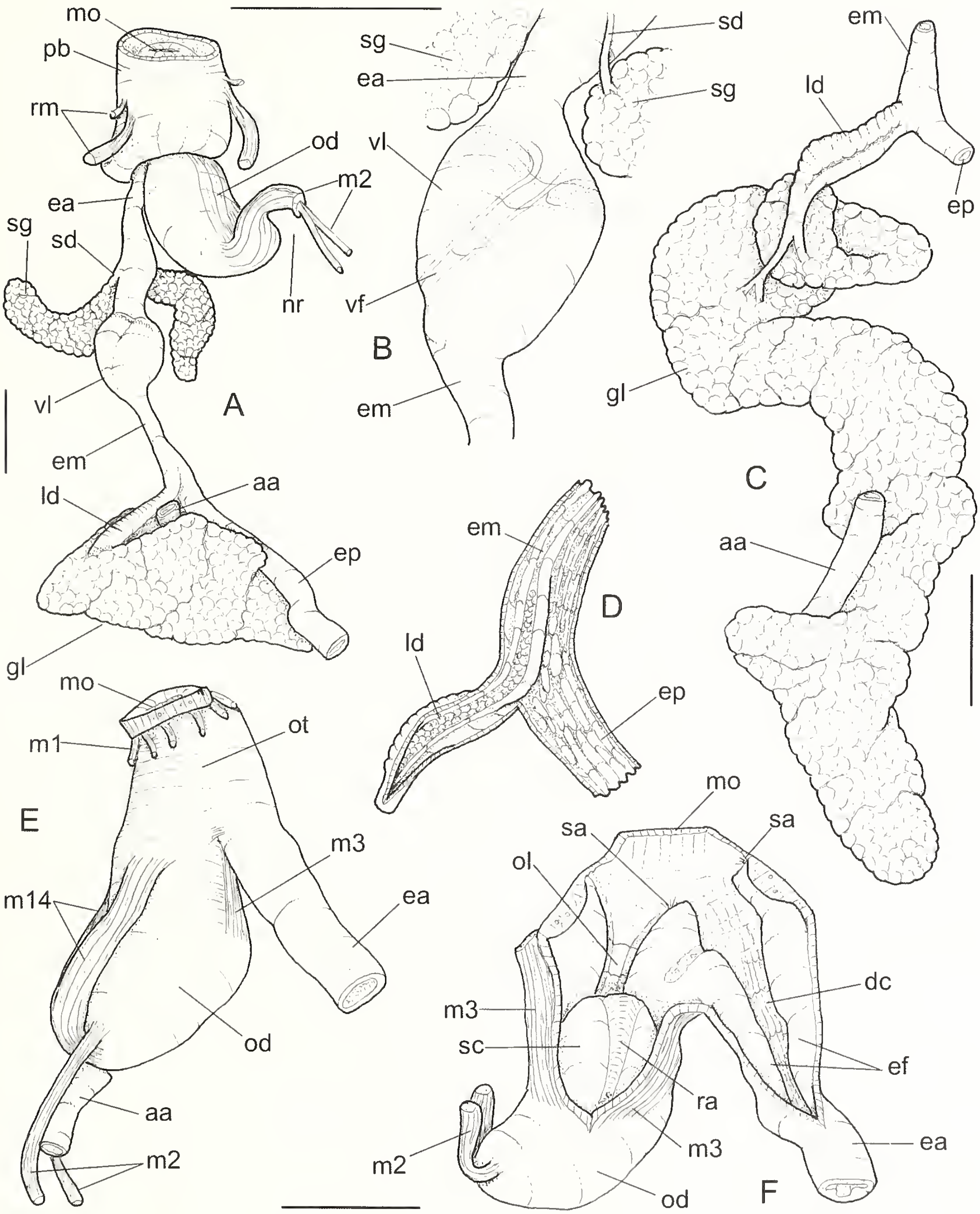
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Figure 7. *Zemira australis* anatomy. A, foregut, extended, ventral view. B, region of the valve of Leiblein, its oblique furrow (vf) seen by transparency. C, gland of Leiblein partially uncoiled, some adjacent structures also shown. D, transition between middle and posterior esophagus and duct of gland of Leiblein, opened longitudinally to expose inner folds. E, buccal mass, left view. F, buccal mass, anterior region opened longitudinally and deflected to expose inner surfaces. Scale bars = 1 mm. Abbreviations listed in section with figure captions.

anterior esophagus (Fig. 7A: em), walls thin; inner surface with longitudinal, low, narrow folds, a pair of close folds larger (Fig. 7D), running towards duct of gland of Leiblein. **Gland of Leiblein** triangular *in situ* (Figs. 5F, 7A), long and somewhat flat if uncoiled (Fig. 7C), becoming about as long as posterior esophagus; anterior aorta crossing between middle and posterior thirds of this gland. Duct of gland of Leiblein long and narrow (about as long as middle esophagus, and about half of its diameter) (Figs. 7A, 7D); having two origins, one sub-terminal in anterior end of the gland, the other in a portion more posterior (Fig. 7C); these two ducts unite within a short distance, remaining duct having pair of tall, longitudinal, narrow folds (continuation from larger folds of middle esophagus); these folds separate a narrow, white, multi-lobed secondary gland from a smooth, narrow area; this secondary gland occupies about 2/3 of duct volume, ending abruptly before the duct's insertion into the esophagus (Fig. 7D). **Posterior esophagus** (Figs. 7A, 7D: ep) about twice as long as the anterior esophagus, inner surface with narrow longitudinal folds, some low, others taller (covering lower folds) diverging and coalescing randomly; these folds disappearing abruptly before stomach. **Stomach** spherical, blind sac, about half the width of adjacent visceral whorl. Esophagus enters stomach along its left anterior region (Figs. 6E, 8A: st), intestine originates to the right of the esophageal insertion. Gastric inner surface (Fig. 8A) mostly smooth, except for a pair of low, narrow folds that run along its left surface, from the esophageal insertion, disappearing gradually into the posterior gastric surface. Duct to digestive gland single, wide, located between esophageal insertion and intestinal origin (Fig. 6E). **Intestine** with tall, dorsal, smooth, long and triangular platform adjacent to the stomach (Fig. 8A); left edge of this platform alongside a band of longitudinal, narrow folds; right edge of this platform serving as insertion of several transversal folds, each about twice as wide as the longitudinal folds, becoming gradually oblique, surrounding ventral surface of intestine, ending at left edge of band with longitudinal folds. Inner surface of intestine, beyond this platform, with only longitudinal folds, very close to each other, filling inner surface totally. Intestine runs almost straight anteriorly, crossing through anterior region of digestive gland, kidney lobe, and right edge of pallial cavity (Figs. 5E, 6E). Rectum and anus described above (pallial cavity).

Genital system. Male (Figs. 5A, 8B). Visceral vas deferens begins a half whorl before anterior end of testis. Within a short distance it becomes a very broad, intensely coiled seminal vesicle, occupying about half of adjacent visceral whorl (Fig. 5D). Seminal vesicle located in the ventral surface of last whorl of the visceral mass, posterior to kidney; becomes narrow at some distance posterior from pallial cavity, running about 1/6 whorl. Prostate gland relatively narrow, running along right edge of pallial cavity ventral to rectum, visceral vas deferens inserting posteriorly (Fig. 5D: pt); walls thick-glandular; no apertures to pallial cavity; inner lumen surrounded by muscle fibers. Prostate spans about ⅓ pallial cavity length, gradually becoming narrow, crossing to pallial floor. This region in the pallial cavity floor with thick, muscular walls, slightly convolute up to penis base (Fig. 8B). Penis slightly larger than half of pallial cavity volume, stubby, dorso-ventrally flat (Figs. 5A, 8B); base broad, with a large, broad right fold covering base of right tentacle; then twisting, remaining tall, flat and thick, narrowing gradually up to bluntly pointed tip (Fig. 8B). Pallial vas deferens within the integument, becoming penis duct. Penis duct running approximately along penis center, very narrow, weakly coiled. Penis aperture apical, very small.

Female (Figs. 8C-F). Visceral oviduct very narrow, running along middle region of columellar surface of the last whorl of the visceral mass, about ¼ whorl preceding pallial cavity, gradually becoming thicker, inserting into pallial oviduct without clear separation. Posterior region of pallial oviduct protruding into kidney, having a narrow zigzag. Albumen (whitish) and capsule (beige) glands adjacent, albumen gland spanning posterior 1/5 of pallial oviduct. Seminal receptacle very small, located between albumen and capsule glands (Fig. 8C); flat to rounded; duct very narrow, attached along the dorsal surface of the pallial oviduct, opening into the vaginal furrow between the albumen and capsule glands. Capsule gland with flat lumen, vaginal furrow running along its left edge, with surface smooth (Fig. 8D). Female pore wide, protruded, with thick edges (Figs. 8E-F: fp). Bursa copulatrix small, short, located along left side of the distal, detached portion of the pallial oviduct (Fig. 8F: bc); with thick muscular walls; its aperture turned anteriorly, occupying about 2/3 of total female pore; inner surface with low, wide, longitudinal folds. Capsule gland aperture narrow, situated to the right of the bursa aperture; its walls thick muscular, protruding inside the chamber of the terminal atrium of the capsule gland. Terminal atrium, with thin walls, located between capsule gland anterior and female pore. Female pore with several wide, longitudinal folds. No cement gland in foot sole.

Central nervous system (Figs. 8G-H). Relatively well-concentrated. No distinction between pleural and cerebral ganglia. Cerebral ganglia broadly connected to each other. Pedal ganglia slightly smaller than cerebro-pleural ganglia; pedal commissure broad, but narrower than cerebral commissure. Cerebro-pedal and pleuro-pedal connectives short, but distinguishable. Pair of buccal ganglia small, located close to posterior edge of the cerebral ganglia.

Measurements of shells (in mm). AMS C333288: ♀1 = 18.6 by 12.0; ♂2 = 19.3 by 12.2; ♂3 = 16.6 by 9.8; ♀4 = 14.1 by 8.7.

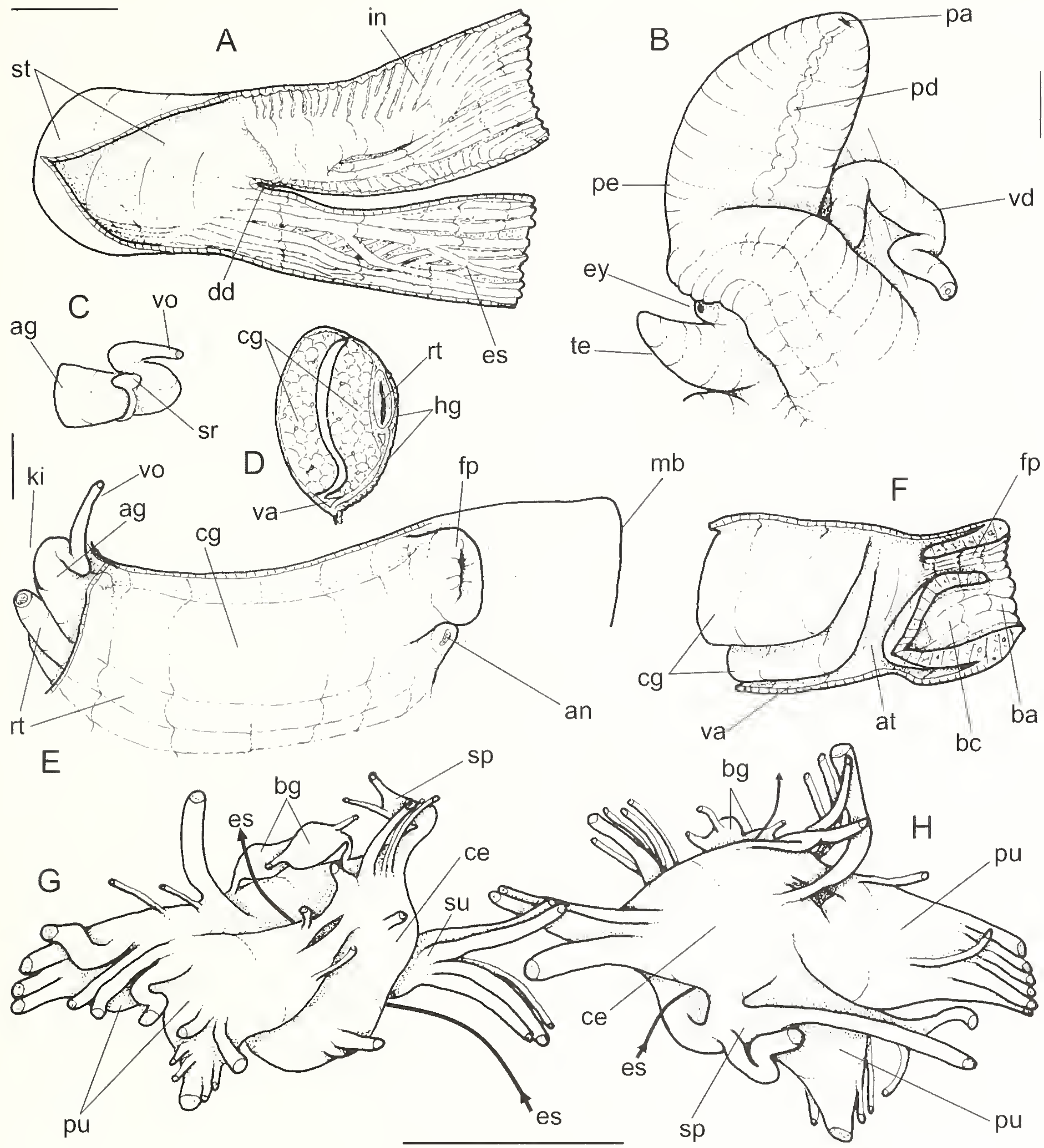

Figure 8. *Zemira australis* anatomy. A, stomach, ventral view, its ventral wall and adjacent region of intestine (in) and esophagus (es) opened longitudinally, inner surface exposed. B, penis and adjacent region of its base, ventral view, penis partially deflected, its duct (pd) seen by transparency. C, pallial oviduct, dorsal view, detail of its posterior region. D, pallial oviduct, transversal section though its middle region. E, pallial oviduct, entire ventral view, including its portion in kidney chamber. F, same, detail of its anterior end, ventral wall removed, region of pore opened longitudinally and deflected to show its parts. G, central nervous system, ventral, slightly right oblique view, esophageal passage (es) shown by arrow. H, same, dorsal, slightly left oblique view. Scale bars = 1 mm. Abbreviations listed in section with figure captions.

Distribution. East coast of Australia.

Habitat. Sandy, from 3 to 146 m depth (Beechey 2005).

Material examined. AUSTRALIA. **New South Wales**; Sydney (Shelf Benthic Survey col.), 1.6 km east of Malabar outlet, 33°58.250'S 151°17.000'E, 66 m depth, AMS C092091, 3♀ (Sta. 027653, SBS3, 26/iii/1973), 2.3 km east of Malabar outlet, 33°59.450'S 151°16.800'E, 66 m depth, AMS C333288, 3♂, 6♀ (Sta. 002609E, SBS5, 25/x/1973), 2.6 km east of Cape Banks, 33°59.500′S 151°16.740′E, 66 m depth, AMS C406792, 1♂ (Sta. 038772, SBS25, 26/i/1973).

Genus *Fulmentum* Fischer, 1884
(Type species *Buccinum sepimentum* Rang, 1832, by monotypy)

Fulmentum ancilla (Hanley, 1859)
(Figs. 9A-E, 4C-D, 10A-13F)

Pseudoliva ancilla: Kantor 1991: 31-34 (figs. 12, 13); Hayes 1994: 77-78.
Sylvanocochlis ancilla: Lorenz 1989: 16-18.
Fulmentum ancilla: Vermeij 1998: 60.

Description

Shell (Figs. 9A-B, 9E). Large (about 60 mm), heavy, biconical. Color brown to beige. Periostracum velvet-like, rust colored, partially eroded. Spire conical, aperture spanning 70% of shell length. Protoconch of 1½ whorls, white, smooth, to weakly reticulated in some areas, border with teleoconch unclear. Teleoconch of 5-6 whorls, each whorl with a weakly concave shoulder. Axial sculpture limited to growth lines. A wide spiral furrow runs along the anterior third of the shell, forming a low, broad labral tooth. Umbilicus absent. Aperture elliptical, posterior end pointed. Siphonal canal wide, open, very short. Inner lip smooth, callus narrow and low except for a small, low node at some distance from posterior end. Outer lip with simple edge, thickened at the shoulder and in region of the labral spine. Thickening of the outer lip and the callus of the inner lip located just posterior to the spiral furrow on the previous whorl.

Head-foot (Figs. 9E, 10B-C, 11A). Head not protruded. Color mostly pale beige, with dark brown, coalescent (forming wide transversal bands) spots in head and dorsal surface of foot (Fig. 9E). Tentacles relatively short, located close to each other; basal half clearly thicker than distal half. Eyes located along outer edge of tentacles at middle level, proximal to their constriction; terminal portion of tentacle very short (Figs. 10B-C). Rhynchostome located ventral to and between the tentacles. Foot spans ½ whorl when retracted; sole simple; anterior edge with deep furrow that contains the pedal glands. Dorsal and ventral edges of this furrow relatively thick and rounded; both ends of anterior edge rounded. Columellar muscle spans ½ whorl, thick; posterior end with a wide, rounded component, and a small (about 10% of origin area) projection located along its left end. Haemocoel about 1/3 head-foot width along its anterior half, posterior half becomes very narrow and turned to the left (Fig. 10C).

Operculum (Figs. 9C-D). Elliptical, horny, brown, occupying entire shell aperture. Nucleus terminal, inferior. Outer surface with concentric growth lines and narrow undulations. Inner surface glossy. Scar occupying about 80% of inner surface, approx. central, slightly displaced closer to inner and superior edges. Scar divided into approx. two similar sized areas by oblique line. Inferior region broadly pointed, with wide free projection; triangular, wide furrow running along middle region of this projection, starting in the apex, finishing in the scar.

Mantle organs (Figs. 10A, 10E). Mantle edge simple, thick, transversally banded. Siphon relatively long (about half of pallial cavity length), edges simple (Figs. 9E, 10A); mantle edge surrounding its base. Pallial cavity with about 1½ whorls. Osphradium about ½ pallial cavity length and about ¼ of its width, with curved, symmetrical filaments; anterior and posterior ends rounded; each filament relatively tall, projecting laterally; dorsal edge connected to mantle along ½ its length the rest supported by a rod; ventral edge thin, with small notch located approximately in central region; right filaments covering ctenidial vein and part of gill filament bases (Fig. 10E: os). Gill about 3/4 of pallial cavity length and 1/4 of its width; anterior end pointed, posterior to that of osphradium; ctenidial vein extending a short distance anterior to gill end; posterior gill end rounded, touching pericardium. Gill filaments triangular and tall, curved to right; rod relatively broad; filament tip rounded, preceded by narrow region. Ctenidial vein narrow, lying along left edge of gill. Afferent gill vessel very narrow, lying at some distance from apparent right gill edge. Hypobranchial gland somewhat tall, whitish, covering entire area between gill and rectum (about ½ pallial cavity width), anterior end at level of anus. Rectum relatively narrow, running along right edge of pallial cavity. Anus detached, situated between middle and anterior thirds of pallial cavity, with small papilla on its right side. Genital ducts lying between rectum and right edge of cavity, described below.

Visceral mass (Fig. 10A). Spans about three whorls. Reno-pericardial structures occupy anterior half whorl. Stomach located just posterior to reno-pericardial area. Digestive gland greenish cream, occupying all whorls, surrounding stomach. Gonad same color as digestive gland, occupying superior and columellar surface of each visceral whorl, terminating a short distance posterior to kidney.

Circulatory and excretory systems (Fig. 10D). Pericardium about ¼ of kidney volume. Heart similar to that of *Zemira*. Kidney somewhat trapezoid, spanning ~½ half whorl. Nephridial gland flat, thicker anteriorly, lying along entire dorsal half of reno-pericardial membrane. Kidney lobe similar to that of *Zemira*. Afferent renal vessel large, with branches running between folds of both kidney lobe flaps, covering right side of nephropore, connected to membrane between kidney and pallial cavities, producing small urinary cavity.

Digestive system (Figs. 11A-12E). Rhynchostome forms a small opening located ventral to and between both cephalic

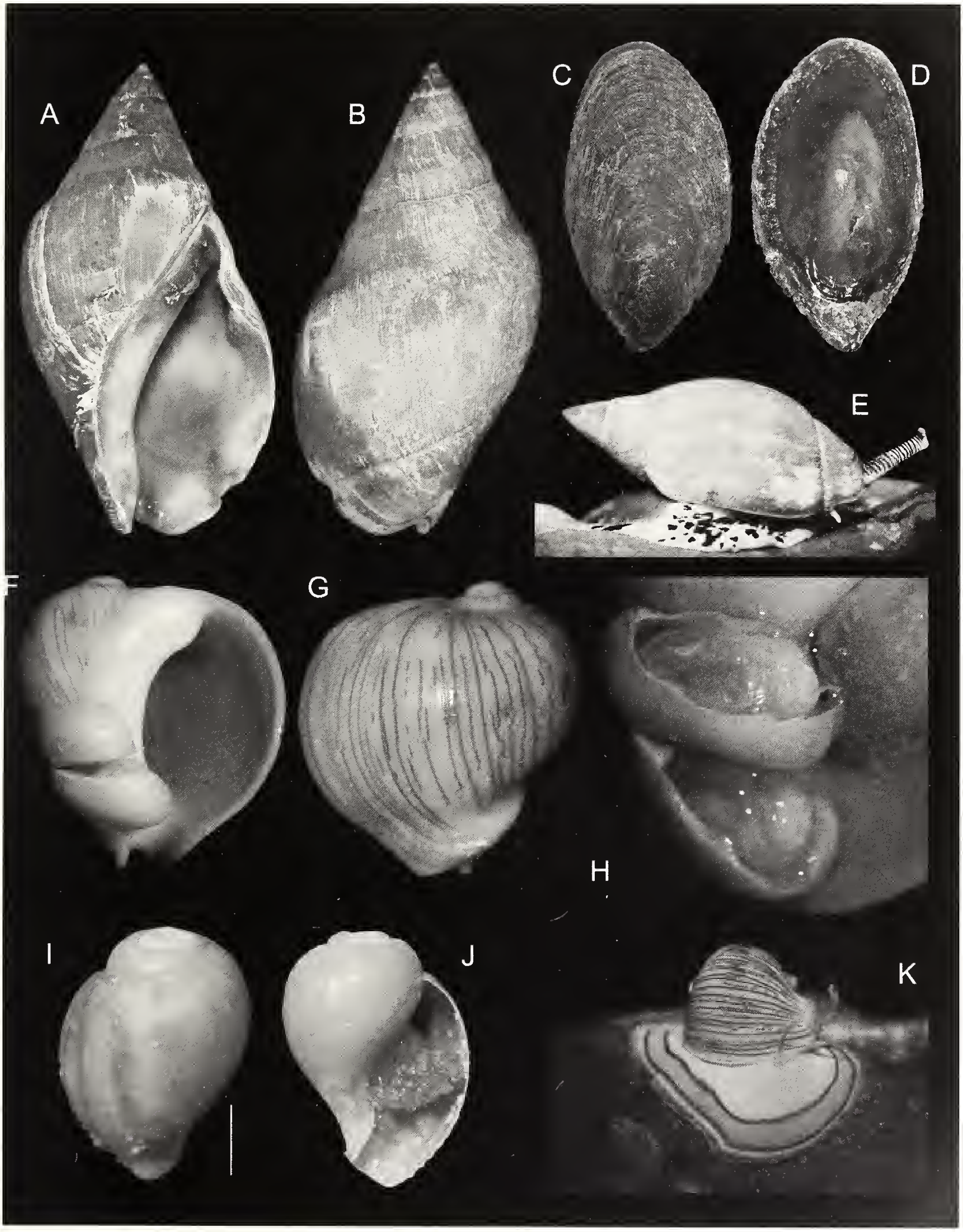

Figure 9. Shells, opercula, and living specimens. A-E, *Fulmentum ancilla*; A-B, shell, NMNH E5279, female, apertural and dorsal views, length = 64.5 mm. C-D, Operculum, outer and inner views. E, Live, crawling specimen from Jeffreys Bay, South Africa, photo courtesy of Brian Hayes. F-K, *Melapium lineatum*. F-G, shell, NMNH V9979, male, apertural and dorsal views, 2 egg capsules attached in anterior region of inner lip (total length = 26,2 mm). H, detail of egg capsules attached to shell, NMNH 59733, portions of both capsules removed, showing young specimens inside. I-J, dorsal and apertural views of a young specimen extracted from an egg capsule shown in the preceding figure, scale bar = 2 mm. K, live, crawling specimen from off Algoa Bay, South Africa, photo courtesy of Brian Hayes.

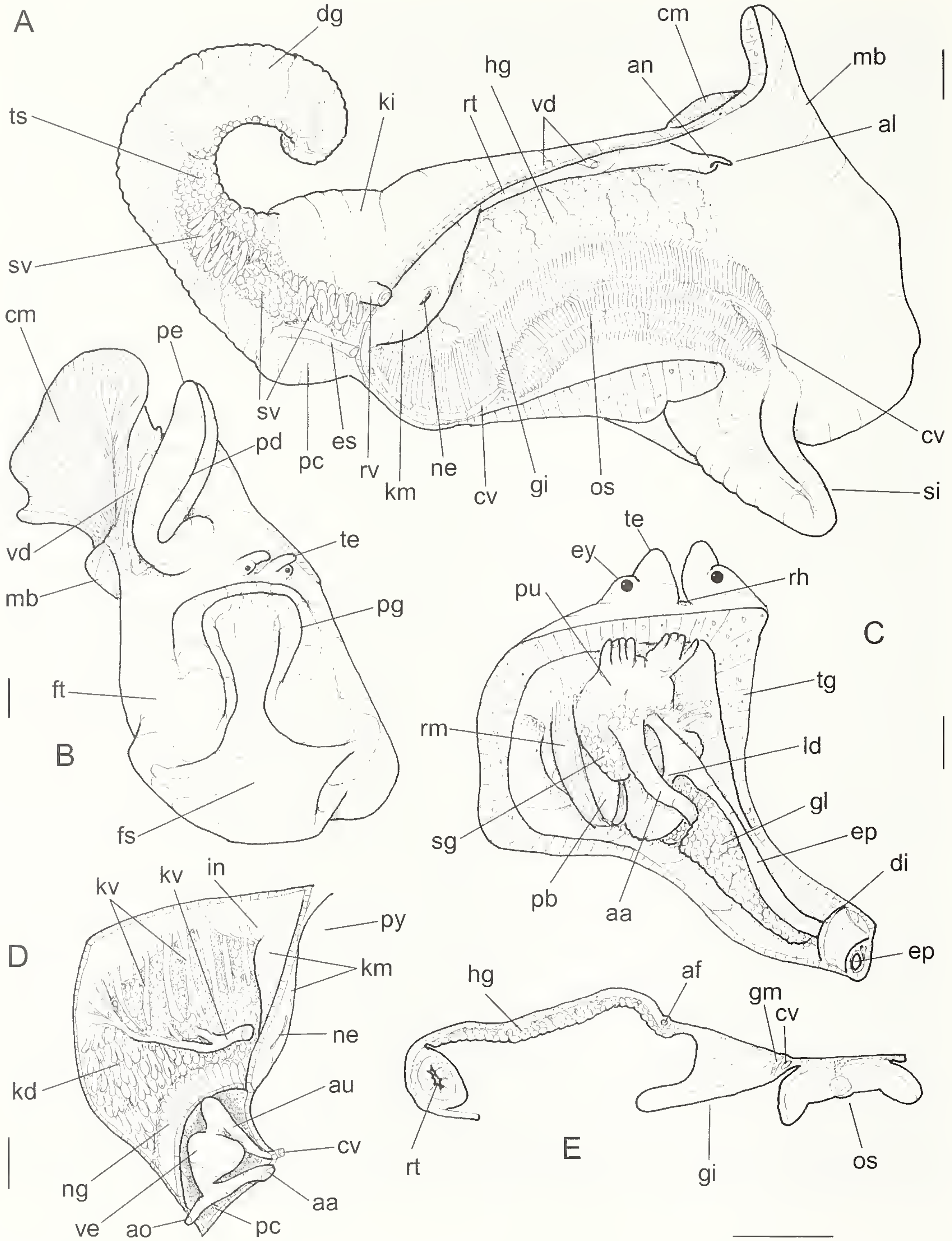
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Figure 10. *Fulmentum ancilla* anatomy. A, pallial cavity and visceral mass, male, ventral-inner view. B, head-foot, male, frontal view. C, head and haemocoel, ventral view, foot and columellar muscle removed. D, kidney and pericardium, ventral view, ventral wall removed, renal membrane with pallial cavity (km) deflected anteriorly (right in fig.). E, Transverse section through pallial cavity roof, at mid-length of the osphradium. Scale bars = 2 mm. Abbreviations listed in section with figure captions.

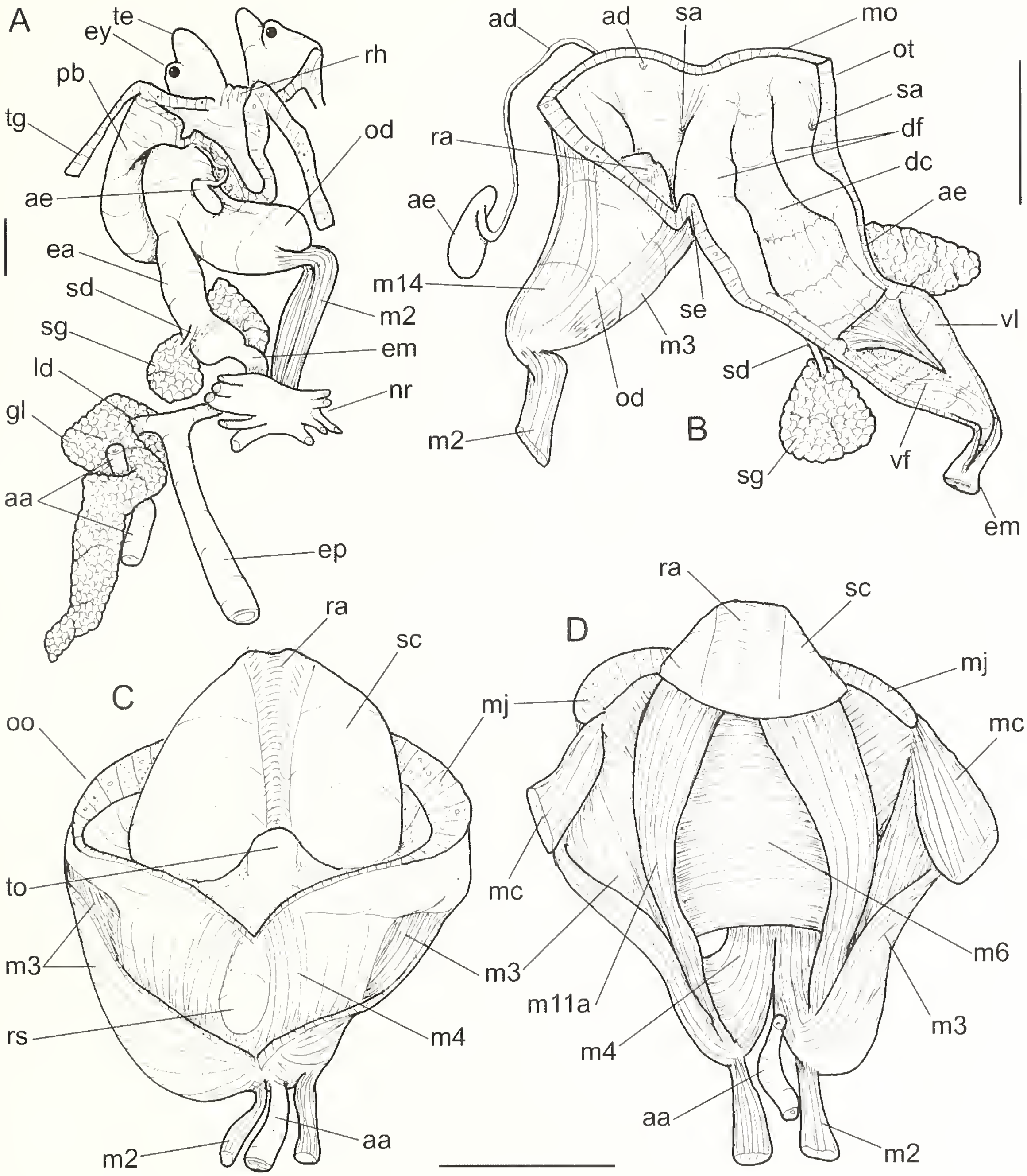
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Figure 11. *Fulmentum ancilla* anatomy. A, foregut partially uncoiled, ventral view, adjacent region of head also shown. B, buccal mass, left view, esophagus opened longitudinally. C, odontophore, dorsal view, some muscles deflected. D, same, dorsal view, superficial layers of muscles and membrane reflected, aorta shown as *in situ*. Scale bars = 2 mm. Abbreviations listed in section with figure captions.

tentacles (Fig. 10C). **Proboscis** short (less than 1/3 of haemocoel length) and broad (about haemocoel width) (Figs. 10C, 11A); walls thick, muscular. Proboscis retractor muscles forming a main pair, one in each side, left retractor muscle slightly more dorsal than right, originating at middle level of haemocoel latero-ventral region, running towards middle level of proboscis, inserting along its distal half (Fig. 10C: rm); several accessory proboscis retractor muscles along dorsal surface, thinner than main retractor muscles, originating in a virtual line connecting both main retractor muscles, surrounding dorsal haemocoelic wall. Mouth a transversal slit on proboscis tip. Oral tube with thick walls, about half the length of the odontophore. Oral cavity wide, with a pair of low, broad, dorsal folds, each occupying about 1/3 of the dorsal surface and 1/3 of area between them; each dorsal fold with rounded anterior end, at short distance from mouth (Fig. 11B); remaining fold characters similar to those of *Zemira*. Ventral chitinous platform present (Fig. 11B, ad). Odontophore about same length as proboscis (when extended), protruding beyond proboscis into haemocoel when retracted (Figs. 10C, 11A). Odontophore organization and musculature similar to that of *Zemira*, with following distinctions. Odontophore muscles (Figs. 11B-12A): **m2** a single pair inserted into posterior region of m4, by side of radular sac; **m2a** absent; **m5** pair wider and thicker; **m6** with anterior end at some distance from that of odontophore cartilages (Fig. 10); **m11a** pair broader (Fig. 11D). Odontophore cartilages elliptical, with posterior region similar to anterior region (Fig. 12A). **Radula** (Figs. 4C-D): **rachidian** tooth with broad base (about 60% of radular ribbon), close to adjacent teeth, with 3 tall (about 1/2 base width), triangular, sharply pointed cusps, somewhat equidistant and separated from each other; distance between rachidian and lateral teeth area equivalent to half of rachidian base width; **lateral tooth** with base 60% of rachidian width, 2 tall (height equivalent to base width), triangular, sharply pointed cusps, one on each side of the tooth, inner cusp slightly smaller than outer cusp. **Anterior esophagus** shorter than odontophore, wall thick muscular, with some muscles connecting its latero-ventral region to the adjacent region of the haemocoel floor, sometimes passing through the salivary glands; inner surface with a pair of lateral folds, as continuations from buccal cavity folds, gradually narrowing (Figs. 11A-B: ae). **Salivary glands** paired, each an elliptical, separated mass located along each side of valve of Leiblein and close to the nerve ring (Figs. 11A-B, 12B: sg). Salivary ducts very narrow, originating in the anterior end of the gland, penetrating the lateral walls of the anterior esophagus within a very short distance; running within this wall up to salivary aperture. Salivary aperture very small, located in lateral edge of buccal cavity dorsal folds, at mid-level, just within the posterior end of a narrow and shallow furrow running anteriorly, surrounding antero-lateral edge of dorsal folds (Fig. 11B: sa). **Accessory salivary gland** single, elliptical, internally hollow, situated within the haemocoel near the middle region of odontophore's ventral surface. Anterior region of gland gradually narrowing without clear division from its duct. Duct long and very narrow, equal to the length of odontophore, lying along ventral surface of the odontophore, odontophore tube and oral tube (Figs. 11A-B: ae); opening of duct very small, in median region of the ventral surface of the oral tube, just posterior to the mouth (Fig. 11B: ad). **Valve of Leiblein** with about half size of odontophore, inner organization similar to that of *Zemira*, with narrow folds of the oblique furrow disappearing gradually at anterior and posterior ends (Figs. 11A-B, 12B: vl). **Middle esophagus**, narrow, roughly equal in length to the anterior esophagus, walls thin, inner surface smooth (Figs. 11A-B: em). **Gland of Leiblein** brown, long, triangular, anterior region wide, narrowing gradually towards posterior, posterior tip narrow and rounded; anterior aorta crossing through this gland between middle and anterior thirds (Figs. 10C, 11A: gl). The portion of the duct of the Gland of Leiblein that is free from the gland is relatively long (about half the length of the middle esophagus) (Figs. 11A, 12C: ld); inner surface with a longitudinal, white gland in its dorsal side, and a smooth, thin region on the ventral side; lacking transverse septa within (Fig. 12C). **Posterior esophagus** narrow, about three times as long as anterior esophagus, wall thin, inner surface smooth or with narrow longitudinal folds, close to each other (Figs. 11A: ep; 12D-E: es). **Stomach** trapezoidal, weakly dorso-ventrally flattened, occupies about half of visceral whorl volume, is situated about 1/4 whorl posterior to kidney (Figs. 12D-E); esophagus joins the stomach at left-anterior end, the intestinal joins the stomach to the right of the esophagus, and is slightly wider. Duct to digestive gland single, located a short distance posterior to esophageal insertion and intestinal origin; duct with very wide and flat base, with branches running from opposite sides after short distance. Gastric walls thick muscular. Gastric inner surface with transversal, low, broad, somewhat irregular folds (Fig. 12E). **Intestine** almost straight, weakly sigmoid, running anteriorly (Figs. 10D, 12D-E); inner surface full of low, narrow, closely spaced longitudinal folds; A larger pair of adjacent folds run along the left side of the stomach, gradually disappearing into rectum. Intestine passes initially through digestive gland, then through the ventral flap of the kidney lobe. Rectum and anus as described above (pallial organs).

Genital system. Male (Figs. 10A-B, 13A-B). Testis as described above (visceral mass). Seminal vesicle very large, occupying the columellar surface of almost the entire last whorl of the visceral mass, forming a relatively narrow, extremely convoluted tube (Fig. 10A: sv). Seminal vesicle abruptly terminates near the pallial cavity, giving rise to a very narrow vas deferens that crosses the afferent renal vessel dorsally, and becomes exposed in the pallial cavity, lying along the posterior and right pallial cavity edges, gradually becoming thicker (Fig. 10A). No prostate gland differentiable. Vas deferens descends to pallial floor at mid length of the pallial cavity. The portion of the vas deferens lying on pallial floor is open (furrow); with tall, thick edges (Fig. 10B), becoming convoluted near the base of the penis. Penis of about 1/4 of pallial cavity volume (Fig. 10B); twisted inwards in basal region, middle region slightly broader, constricting gradually up to narrow, rounded tip. Penis duct

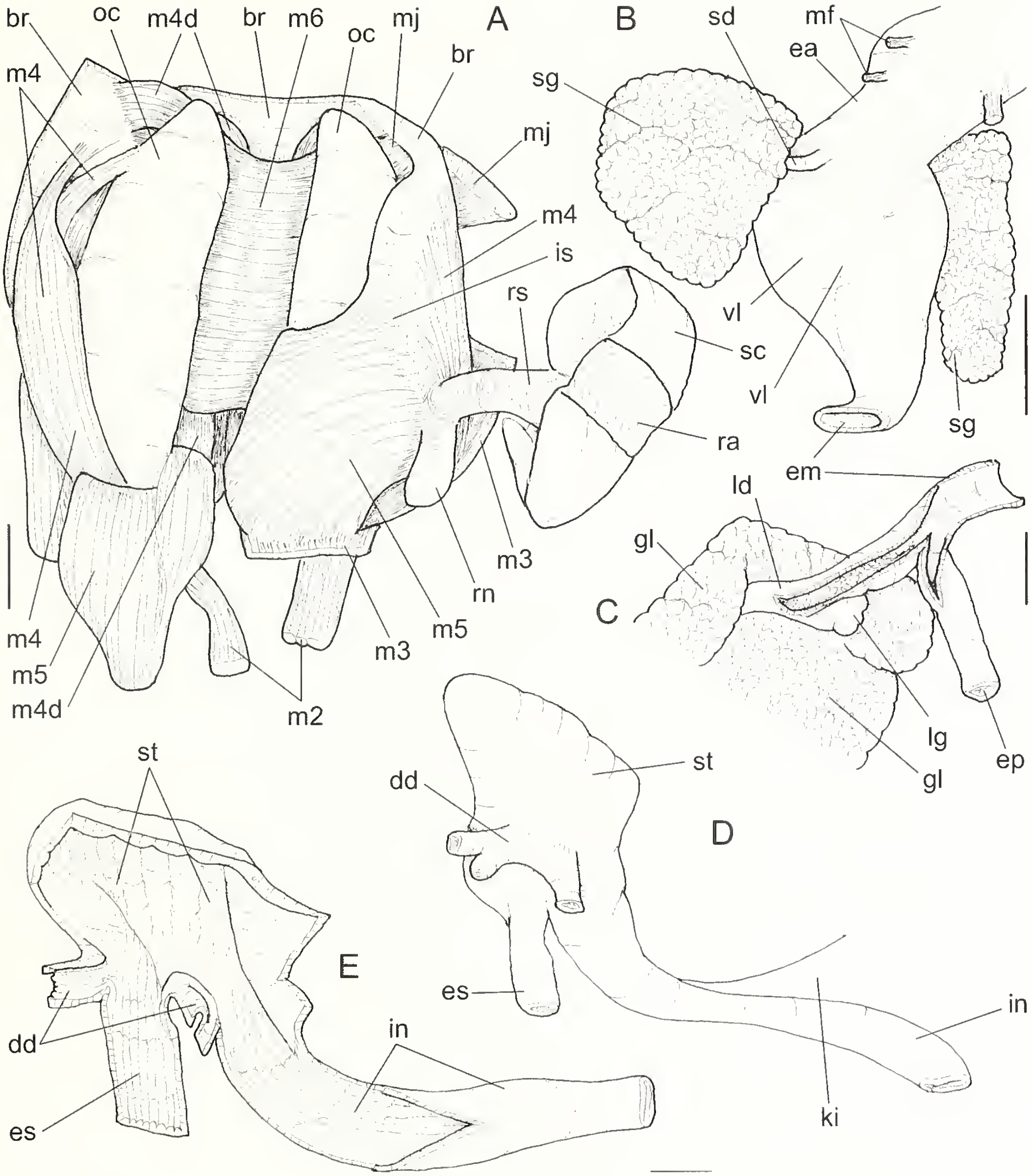
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Figure 12. *Fulmentum ancilla* anatomy. A, odontophore, dorsal view, most of the superficial membrane and muscles removed, radular sac deflected to right, both cartilages (oc) deflected from each other, left m5 turned downwards. B, region of the valve of Leiblein (vl), ventral view. C, region of duct of the gland of Leiblein (ld), ventral view, most tubes opened longitudinally. D, midgut as *in situ*, ventral view, position of kidney indicated. E, same, most tubes opened longitudinally to expose inner surface. Scale bars = 1 mm. Abbreviations listed in section with figure captions.

open (a furrow), lying on inner penis edge, relatively deep, runs up to penis tip (Fig. 13A). Terminal papilla in penis tip, about 1/6 of penis length, located inside a chamber formed by terminal portion of penis (Fig. 13B).

Female (Figs. 13C-D). Visceral structures similar to those of males. Visceral oviduct very narrow, running along the middle of the columellar surface of the visceral mass. Visceral oviduct inserting in left side of pallial oviduct, an-

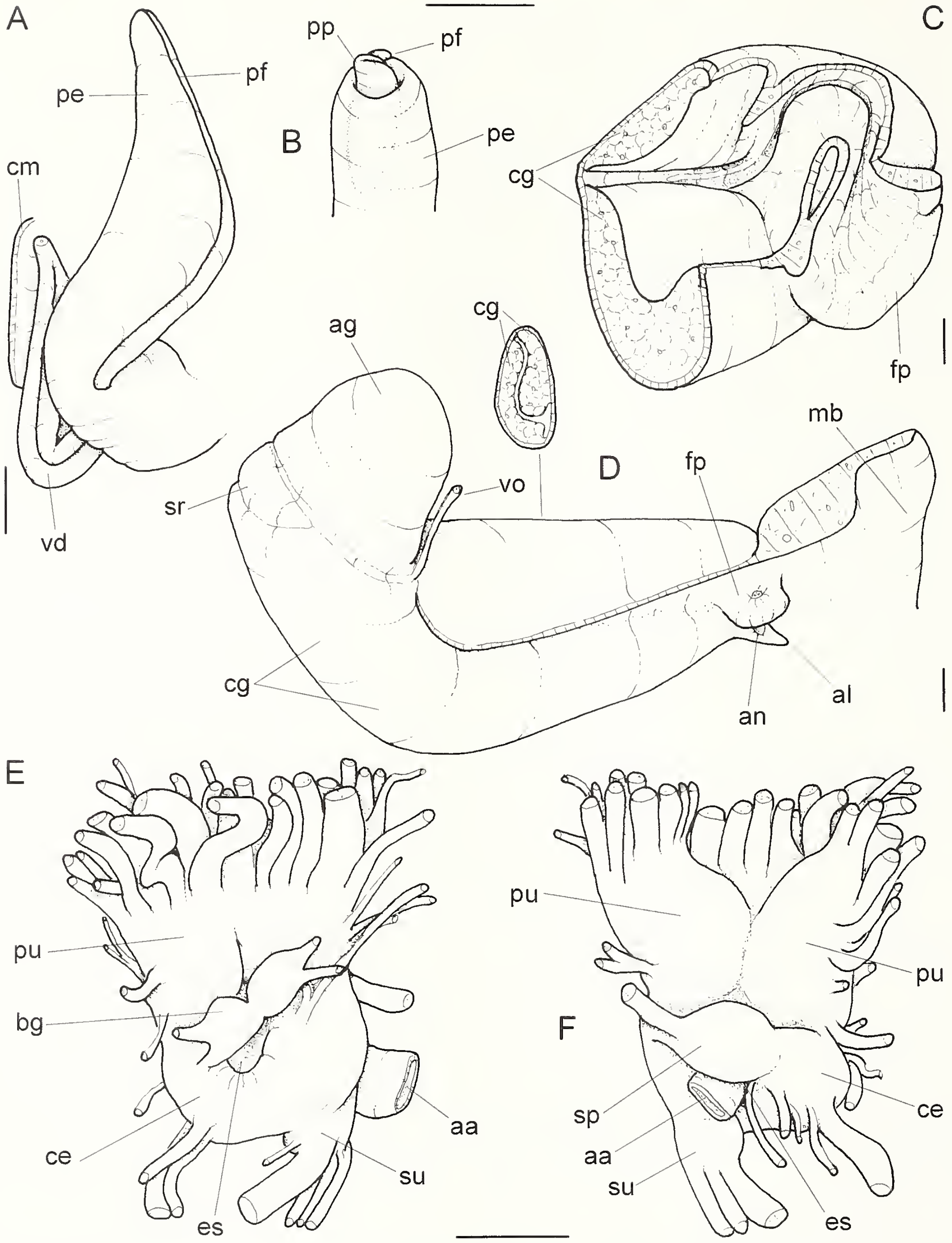
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Figure 13. *Fulmentum ancilla* anatomy. A, penis and adjacent region of its base, dorsal view. B, detail of penis tip, showing, partially by transparency, terminal papilla. C, pallial oviduct, ventral view, detail of its terminal region mostly opened longitudinally, walls reflected. D, entire pallial oviduct, ventral view as *in situ*, some adjacent structures also shown, a transverse section at indicated level also shown. E, central nervous system, ventral view, esophageal passage indicated (es). F, same, dorsal view. Scale bars A, D = 2 mm; others = 1 mm. Abbreviations listed in section with figure captions.

terior to albumen gland, opening to vaginal duct. Albumen gland whitish, about ¼ length of pallial oviduct, forming a blind-sac with thick walls and flat lumen that is continuous with the lumen of the capsule gland. Seminal receptacle triangular, located between albumen and capsule glands, close to right-dorsal side, no apparent duct, opening directly to vaginal duct between both glands. Capsule gland beige, inner lumen broad and flat (as wide as gland). Vaginal duct lying all along capsule gland left edge, separated from it by a low fold of dorsal lamina of capsule gland. Capsule gland laminae terminating close to genital pore, without forming a vestibule. Female pore protruded, rounded, located to right of anus. Female pore walls thick muscular, inner lumen flat, curved from left to right, then to left again, expanding gradually, leading to pore (Fig. 13C). Female pore edges thick, preceded by longitudinal, broad folds. No cement gland in sole of foot.

Central nervous system (Figs. 13E-F). Located at base of proboscis, a short distance ventral to the rhynchostome. Very concentrated, practically no individual ganglia distinguishable. Cerebro-pleural ganglia widely connected to each other along median line. Both also widely connected to pedal ganglia, no connective distinguishable. Pedal ganglia of about the same size as the cerebro-pleural ganglia. Commissure between both pedal ganglia relatively narrow and very short (both pedal ganglia maintained in contact). Pair of buccal ganglia small, close to each other, located obliquely (left ganglion slightly more anterior) in dorsal region of cerebro-pleural ganglia; connective with cerebral ganglia narrow and short, left connective a little longer and joining another secondary nerve, running anteriorly. Supra- and subesophageal ganglia about half the size of the main ganglia, located near and ventral to right cerebral ganglion; subesophageal ganglion connected to cerebral ganglion by a narrow and short connective; supra-esophageal ganglion connected to subesophageal ganglion by a broad and very short connective, and also to left cerebral ganglion by a narrow and short connective.

Measurements of shells (in mm). NMSA E5279: 64.5 by 35.0.

Distribution. South Africa.

Habitat. Rocks and coarse sand, from 32 to 81 m depth.

Material examined. SOUTH AFRICA. **Western Cape**; 93 km SE of Mossel Bay, 68.4 m depth, NMSA E2770, 1♀ (no shell) (Exch. C. Marais col., 24/vi/1988), SW of Mossel Bay, Agulhas Bank, 81 m depth, NMSA E5279, 1♀ (Exch. C. Marais col., xi/1988); Struis Bay, 34°47.2′S 20°08.6′E, 32 m depth, NMSA S3578, 1♂ (no shell) (dredged Sardinopsis, 08/vi/1991).

Discussion. *Fulmentum ancilla* has been mostly referred in the genus *Pseudoliva* or *Sylvanocochlis* Melvill, 1903, for which it is type species. Recognition of *Fulmentum* is based on the arguments of Vermeij (1998: 60), who considered both genera (*Sylvanocochlis* and *Fulmentum*) as synonyms. Kantor's (1991) anatomical description was used as the ground plan for the anatomical study of this species. Results of the present study generally agree with Kantor's data; the few different points include the presence of transversal distinct folds in the hypobranchial gland found in his specimens (his fig. 12D), but lacking in those examined here.

Genus *Melapium* Adams and Adams, 1853
(Type species *Pyrula lineata*, by subsequent designation of Cossmann, 1901)

Melapium lineatum (Lamarck, 1822)
(Figs. 9F-K, 4E-F, 14A-17F)

Melapium lineatum: Liltved 1985: 9; Kantor 1991: 39-41 (figs. 1A-B, 2B, 17, 18); Hayes 1994: 77-78; Vermeij 1998: 75.

Description

Shell (Figs. 9F-K). Of medium size (about 30 mm), rounded; wider than long. Walls thick. Color cream, with narrow axial bands, dark beige, with irregularly intercalated longer and shorter bands, mainly concentrated in a band along the middle of the body whorl; canal white, with purple pigmentation within the anterior edge. Protoconch flat, dome-shaped, of 1½ whorls; transition to teleoconch indistinct (Figs. 9G-J). Spire flat, low, weakly elevated, with about 3 whorls. Suture relatively deep. Body whorl very large, surrounding almost completely the penultimate whorl. Surface glossy, lacking sculpture except for weakly visible growth lines. Anterior region with carina surrounding left edge of canal, projected forwards. Aperture rounded (Fig. 9F), located close to suture, peristome white, gradually becoming orange in interior regions. Outer lip simple, semi-circular; edge thick, rounded. Inner lip bearing thick callus, covering roughly half of the ventral surface. Canal short, broad, relatively deep, projected forwards. Young specimens (about 2 whorls) antero-posteriorly longer, outline elliptical (Figs. 9I-J); outer surface opaque, sculptured by a net of thin and very narrow reticulation of spiral and axial lines (Fig. 9I).

Head-foot (Figs. 9K, 14A-B, 14 D, 15B). Head weakly protruded, socket-like; basal region of head as short flap. Tentacles located in both ends of this flap; each tentacle long and narrow, with a broader region just above base; tip pointed. Color mostly beige-cream, with bluish band flanked by a narrow bands of red and yellow surround the dorsal surface of foot at its margins (Fig. 9K); tentacles with pale base and orange middle and distal regions. Eyes located on both ends of head-flap, below the base of each tentacle (Figs. 14A, 14D). Rhynchostome in the form of a transversal slit is located between the tentacles (Fig. 14D). Foot very wide and broad; thick in center, gradually becoming thinner toward the periphery, uniformly in all directions. Furrow of

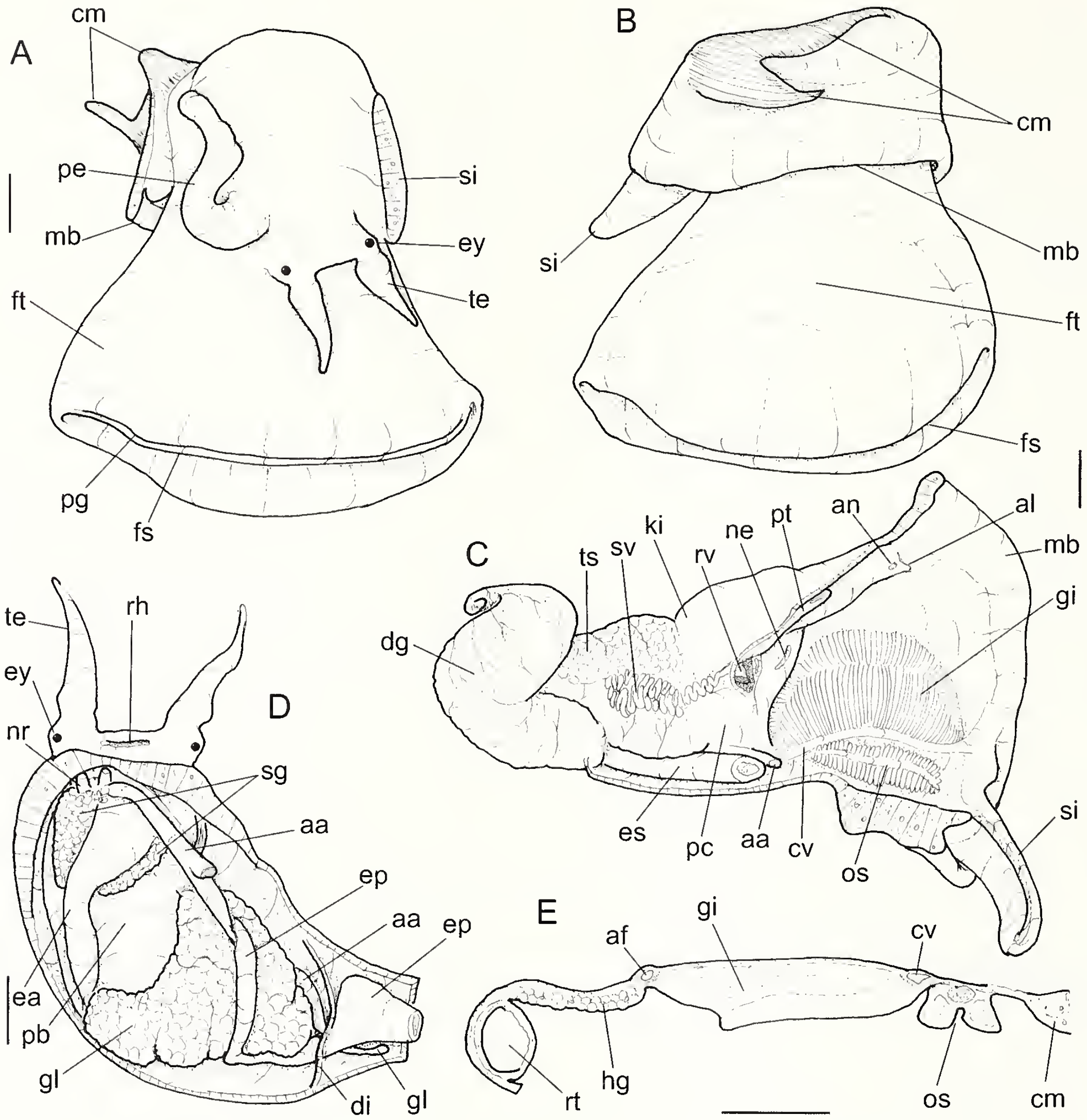

Figure 14. *Melapium lineatum* anatomy. A, head-foot, male, frontal view. B, same, posterior view. C, pallial cavity and visceral mass, male, ventral-inner view. D, head and haemocoel, ventral view, foot and columellar muscle removed. E, Transverse section through pallial cavity roof, at mid-length of the osphradium. Scale bars = 2 mm. Abbreviations listed in section with figure captions.

pedal glands very thin, restricted to anterior half of foot edge (Fig. 14A: pg). Columellar muscle of about 1/3 whorl, having broad main flap in middle and right regions; secondary flap taller and longer, at its left end, projected deeper, weakly coiled (Fig. 12A: cm). Male penis relatively small, originating far removed posteriorly from right tentacle (described below). Haemocoel oval, broad, weakly curved to left (Fig. 14D).

Operculum. Absent.

Mantle organs (Figs. 14C, 14E). Mantle border simple, wide, thick, unpigmented. Pallial cavity broad and short (just over ½ whorl). Siphon long (equal in length to pallial cavity) and slender; edges simple; inner surface with low transversal folds. Siphon base with pair of reinforcements extending as low folds beyond siphon base, parallel to mantle border, longer on right, about 1/3 of mantle border length, gradually diminishing. Osphradium slightly longer than ½ pallial cavity length and about 1/5 of its width; anterior end rounded, posterior end pointed. Osphradium filaments tall, symmetrical, mostly free from attachment to mantle roof (connected mainly to osphradial ganglion), forming a longitudinal concavity along this ganglion. Each filament extends ventrally about twice the diameter of the osphradium ganglion, its edge reinforced by a rod that is weaker along inner regions closer to the ganglion. Gill elliptical and broad, slightly shorter than pallial cavity and about half as wide, slightly curved to left. Anterior and posterior ends broadly pointed, anterior end slightly forward of osphradium margin, and separated from it by a low, broad fold of the siphonal base. Posterior end of gill extends beyond posterior end of osphradium, reaching the pericardium. Gill filaments triangular, relatively low, apex at or slightly to the right of center, rounded; rod broad, lying along left edge, extending slightly beyond the membranous portion of the filament. Ctenidial vein and afferent gill vessel narrow (afferent slightly narrower), running along respective gill edges. Hypobranchial gland moderately thick, cream colored, covering most of the area between the gill and rectum (~1/3 of pallial roof), becoming narrow in the region anterior to the anus, surrounding right edge of gill. Rectum relatively broad, running along the right edge of the pallial cavity for about 2/3 of its length. Anus detached, located between middle and anterior thirds of pallial cavity; with small papilla along its left edge. Genital ducts lying between rectum and pallial floor, described below.

Visceral mass (Fig. 14C). Of about 2½ whorls, rapidly enlarging and almost involute. Right portion of visceral structures encroaching into the right posterior portion of the pallial cavity. Kidney triangular, spanning ~½ whorl along its right border, partially located inside the middle and right portion of the posterior pallial cavity. Pericardium located slightly to the left of the middle of the posterior portion of the pallial cavity. Stomach located about 1/3 whorl posterior to kidney, occupying about half of adjacent visceral whorl volume. Digestive gland orange, extending from apex to kidney, surrounding middle digestive tubes. Gonad within right and columellar surfaces of visceral mass, of the same color as the digestive gland all along its length. Ovary surrounded entirely by digestive gland, becoming internal to it.

Circulatory and excretory systems (Figs. 14C, 15A). Heart volume about 1/3 kidney volume; characters similar to those described for *Zemira*, auricle small, entirely attached along antero-dorsal region of pericardium; ventricle very large, intensely muscular. Anterior aorta 5-6 times thicker than posterior aorta. About half of the kidney encroaches into the pallial cavity roof. Nephridial gland relatively thick (thicker anteriorly), covering entire middle and dorsal region of reno-pericardial membrane. Renal lobe similar to *Zemira*, but with more developed transverse, irregular folds; ventral and dorsal flaps of renal lobe with tall folds intercalating with each other successively along their right region. Rectum passes through ventral flap of renal lobe. Dorsal flap of renal lobe hard and yellowish; ventral flap whitish and softer. Renal efferent vessel very large (almost as large as anterior aorta); running from posterior end of haemocoel, penetrating intro renal chamber with left side broadly attached to middle region of pericardium, before giving rise to multiple branches that become progressively smaller and insert between renal lobe folds. Nephropore a transversal slit, with muscular edges, located in middle of reno-pallial membrane; internally free from folds or vessels inserting close to it.

Digestive system (Figs. 15B-16E). **Proboscis** relatively short (about 1/3 of haemocoel length) and narrow (about half of its width) (Figs. 14D, 15B). Several narrow proboscis retractor muscles surround it almost completely, but more concentrated in dorsal-right region, originating in anterior and middle surfaces of haemocoel (Fig. 15B). Proboscis wall very thick in rhynchodeal region, relatively thin in buccal mass region (Fig. 15D). **Mouth** transverse in proboscis tip. **Oral tube** relatively long (about same length as odontophore) and narrow; inner surface with a broad pair of dorsolateral folds, each fold with about 1/3 of oral tube surface, anterior end of each fold rounded, narrowing gradually posteriorly. A shallow area between both folds is equivalent to that of each fold in width (Figs. 15D-E). A longitudinal platform runs along the ventral surface medially, flat, thick, with weakly elevated lateral edges; remaining surface of oral tube smooth (Fig. 15E: ol). Odontophore tube forms extension of the oral tube (Fig. 15E: oo), ventral platform runs along it up to odontophore; walls thin muscular; length equivalent to that of odontophore.

Odontophore oval, about 1/3 of proboscis length. Odontophore organization and muscles somewhat similar to those described for *Zemira*, distinctions are as follows: Odontophore muscles (Figs. 15E-16D): **m2** pair narrow and thin, also inserting in m4 posterior region; **m2a** absent; **mc** pair very wide, inserting in ventral surface of cartilages surrounding at some distance the m6 insertion (Figs. 16A-C); **m3l**, a thin layer of longitudinal muscles covering the dorsal surface of the odontophore tube from the antero-dorsal edge of the odontophore to the intersection between the odontophore tube and esophagus; **m3t**, a thin layer of transverse muscle fibers covering posterior and ventral surfaces of odontophore (Fig. 16B), posterior to m3a; **m4** pair thinner, originated from posterior edge of cartilages, with fibers splayed to form fan, surrounding cartilages inner surface; **m5**, weakly differentiable from m4, appearing as median continuation of m4; **m6**, thin and relatively narrow, anterior region at level of anterior end of cartilages, posterior end about at middle level of cartilages, inserting into ventromedial edge of cartilages, except in posterior region where they become gradually wider, inserting in outer cartilages

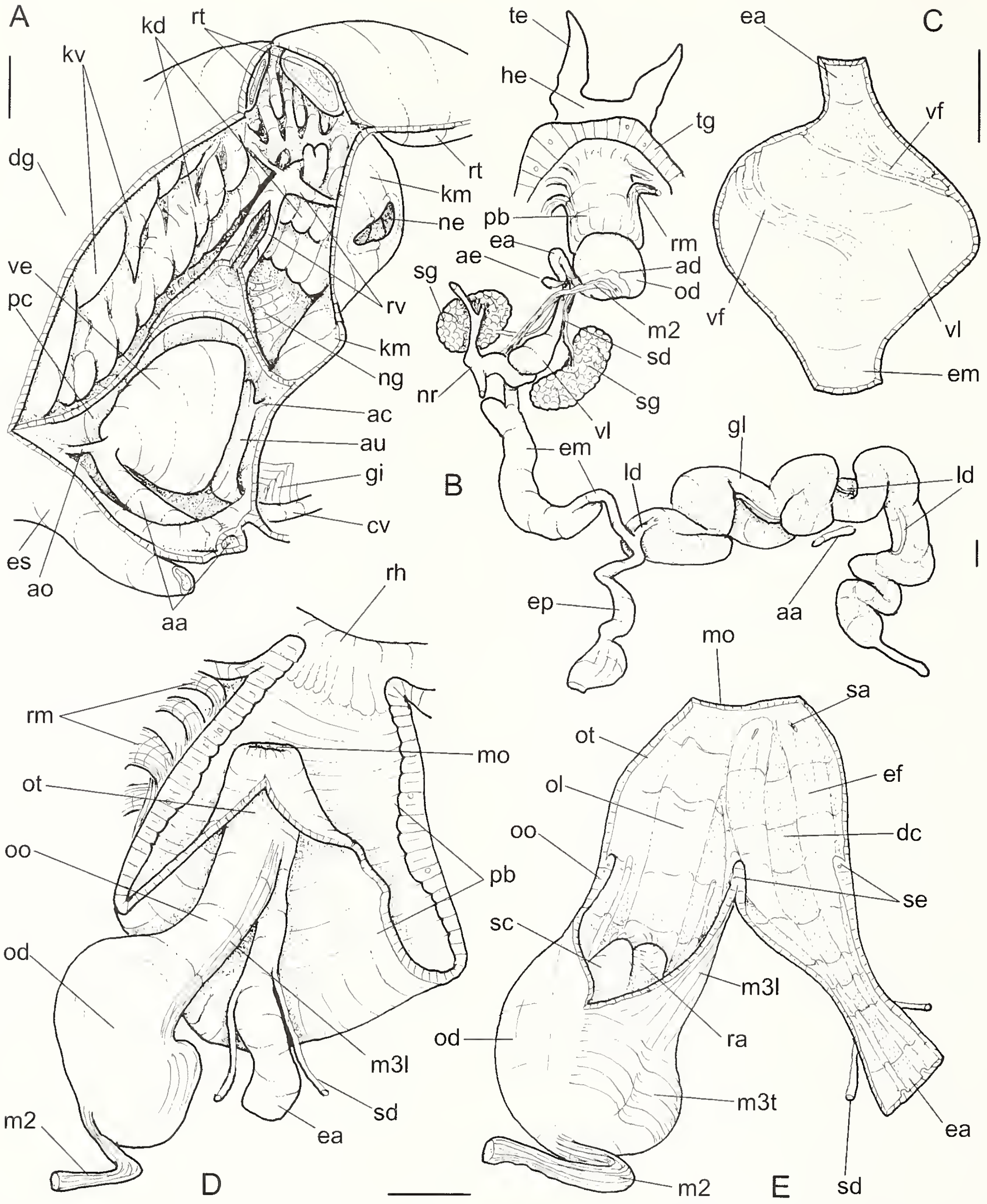
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Figure 15. *Melapium lineatum* anatomy. A, anterior region of visceral mass, ventral view, pericardial and renal ventral wall removed, efferent renal vessel (rv) opened longitudinally, rectum (rt) sectioned transversally. B, head and foregut, ventral view, most structures partially uncoiled. C, valve of Leiblein opened longitudinally, its inner structures (gland and valve) removed, only its oblique furrow (vf) remaining. D, proboscis and buccal mass, left view, proboscis opened longitudinally. E, buccal mass, left view, esophagus and odontophore tube (oo) sectioned longitudinally. Scale bars = 1 mm. Abbreviations listed in section with figure captions.

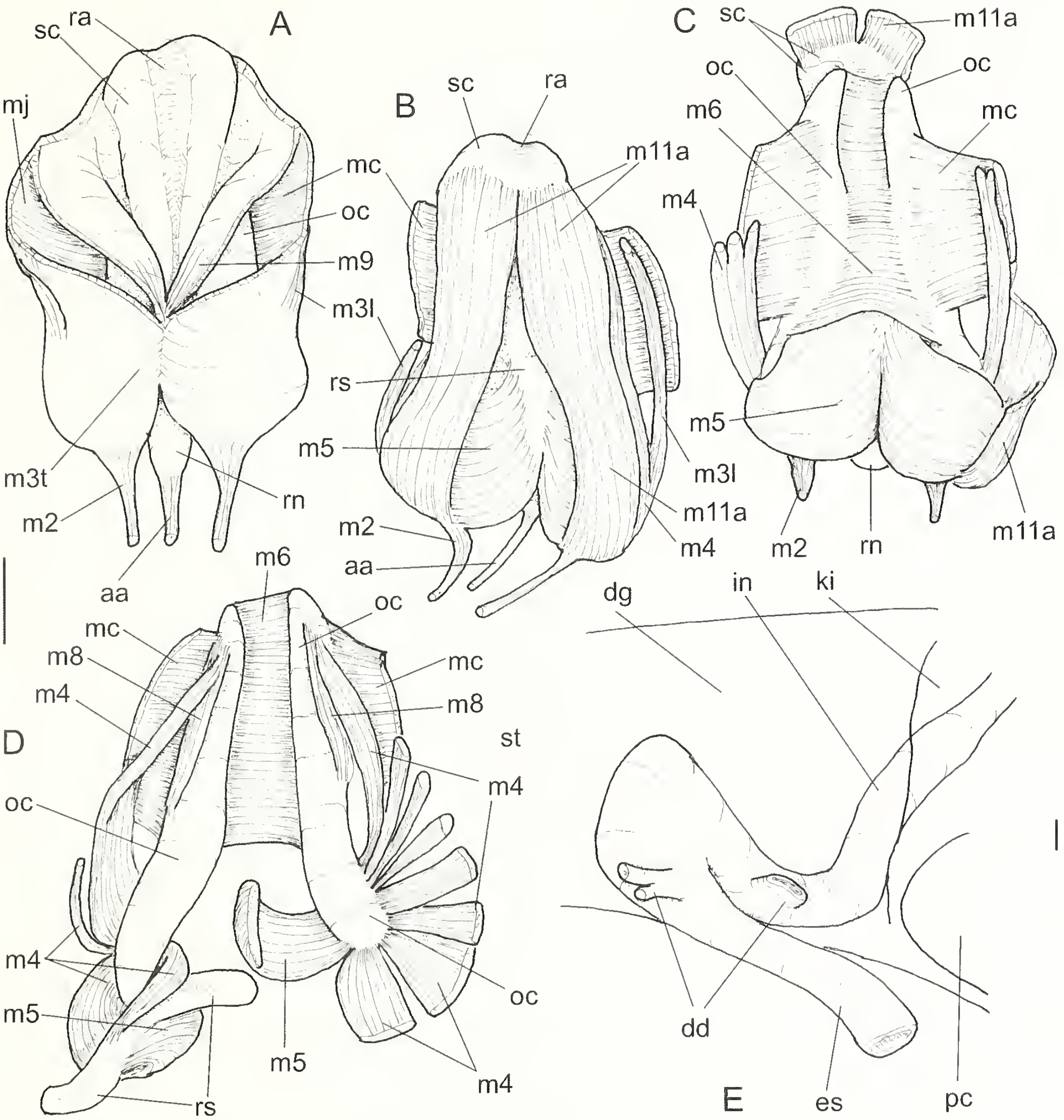
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Figure 16. *Melapium lineatum* anatomy. A, odontophore, dorsal view, odontophore tube connecting it to the oral tube opened longitudinally. B, same, ventral view, superficial layer of membrane and muscles removed, circular muscle (mc) sectioned and reflected. C, same, ventral tensor muscle (m11a) sectioned and reflected. D, same, ventral view, radular sac totally removed and reflected downward and to the left, both cartilages and most muscles reflected. E, midgut as *in situ*, ventral view, topology of adjacent structures also shown. Scale bars = 1 mm. Abbreviations listed in section with figure captions.

surface (Figs. 16C-D); **m8**, pair of short muscles on dorsal edge of cartilages (mostly probably part of m4), running attached to dorsal-anterior edge of cartilages, from their anterior region to their middle level (Fig. 16D); **m11a** pair very wide and thin, originating on m4-m5 posterior region (Fig. 16B). Odontophore cartilages (oc) thin and anteroposteriorly long; anterior end broadly pointed; posterior end rounded, uniform in width along their length (Figs. 16C-D). Subradular cartilage (Fig. 16A: sc) relatively narrow, covering only part of odontophore portion exposed in buccal cavity. Radular sac relatively short, slightly longer than odontophore. **Radula** teeth (Figs. 4E-F): **rachidian** tooth wide, spanning 70% of radular width, base wide, boomerang-shaped, with lateral ends rounded, broader than central area, somewhat arched, with 3 pointed, closely spaced, triangular cusps equal in size located in central area, with length equivalent to transverse width of base. Cusps aligned and joined in a single, projected, flat base. Space between rachidian and lateral tooth narrow. **Lateral** tooth hook-like, base about ¼ of rachidian width, gradually narrowing and curving inwards distally, base disposed somewhat obliquely, tip sharply pointed. **Anterior esophagus** originating anterior to odontophore tube, separated from it by a tall septum; inner surface with pair of lateral folds (continuation from folds of oral tube), smooth (Figs. 15B, 15E: ea); three times the length of the odontophore. **Salivary glands** relatively large, as two separated masses, clustered around valve of Leiblein and nerve ring (Figs. 14D, 15B). Salivary ducts very narrow, originating in middle of each gland's median surface, running anteriorly close to anterior esophagus, penetrating the anterior esophageal walls at some distance from oral tube, at the level of the odontophore; then both ducts run within the esophageal walls and inside the dorsal folds of the oral tube; salivary aperture small, in middle region of dorsal folds anterior end (Fig. 15E: sa). **Valve of Leiblein** with about 1/4 of odontophore volume, inner organization similar to that of *Zemira*, but differs in the oblique furrow being wider and shorter, undergoing the entire torsional rotation within the valve walls, ending just beyond the middle portion of the valve and at the same level as it started in region preceding valve (Figs. 15B-C). **Middle esophagus** (Fig. 15B: em) broad, about twice the length of the anterior esophagus, internally 3/4 filled by a whitish gland that lacks an inner septum. This gland lies along a region of esophagus, its anterior and posterior ends forming short blind-sacs that are located at some distance from the anterior and posterior ends of the middle esophagus.

Beyond this gland is a hollow furrow that is separated from the glandular region by a pair of tall and narrow folds. These folds connect with the bases of the blind sacs at both ends of the gland. The remaining middle esophagus (anterior and posterior to gland) a simple tube with thin walls [Kantor (1991: 39) referred to this gland as accessory gland]. **Gland of Leiblein** long and irregularly coiled, somewhat flat (Fig. 15B: gl); width roughly uniform along its length, except for a broader anterior region close to its juncture with the esophagus and very slender, hollow, distal region, with a small, rounded tip. Duct of the gland of Leiblein runs all along its inner surface (Fig. 15B: ld), with a short proximal portion of the duct free of the gland, its inner surface simple, with 7-8 narrow, longitudinal folds. **Posterior esophagus** as long as middle esophagus, anterior half narrow, inner surface smooth, expands abruptly after passing through the diaphragm dividing the haemocoel from the visceral cavity (Fig. 15B: ep), developing 10-12 tall, glandular longitudinal folds, somewhat separated from each other. **Stomach** occupies about half of the whorl volume, forming inflated curve (Fig. 16E); two ducts to lead to the digestive gland, the duct closer to the esophageal insertion is narrow, bifurcating at a short distance from its origin, turned postero-ventrally; the other duct is closer to the intestine, broad, longer antero-posteriorly, turned antero-ventrally. The inner gastric surface is mostly smooth, except some low folds continuing from the posterior esophagus that gradually diminish posterior to esophageal duct to digestive gland. Intestine (Fig. 16E: in) slightly broader than the esophagus, initially running parallel to it. There is no clear demarcation between the intestine and the stomach. In a short distance, the intestine broadens to become as wide as the stomach and curves abruptly toward the right, lying on the posterior renal wall (Fig. 16E). Rectum and anus as described above (pallial organs).

Genital system. Development (Figs. 9F, 9H, 17C). There is evidence to suggest direct development, as most specimens, including males, carry a pair of large egg capsules attached to the anterior region of inner lip (Fig. 9F). Both capsules are equivalent in size and each contains a single specimen with 3 or more whorls (Fig. 9H). Each capsule is long, somewhat cylindrical, with both ends rounded. The capsule wall is flexible, heavy, thin but strong, pale beige in color, and opaque, not transparent. Capsules are attached by a short and wide peduncle, located longitudinally along the side (Fig. 17C). Both capsules remain side by side, situated transversally across the anterior half of the shell's inner lip.

Young specimens removed from capsules show complete development, no operculum and appear to have the capacity for crawling (Figs. 9H-J). Nothing but viscous yolk was found inside capsules, except occasionally a granulose soft tissue surrounding young specimens in early development (Fig. 17C).

Male (Figs. 14A, 17B). Testes located along the columellar surface of the visceral whorls. Seminal vesicle situated ~½ whorl posterior to pallial cavity, consists of a highly convoluted narrow tube that is ⅓ the width of the visceral mass width and runs along the middle, columellar region (Fig. 14C: sv). A narrower region of the seminal vesicle adjacent to the pericardium and afferent renal vessel opens into the middle region of the posterior-ventral end of pallial cavity. Pallial vas deferens gradually becomes open and glandular forming the prostate gland. Prostate gland narrow, opened as a thick walled furrow, running along ventral surface of rectum for about ½ pallial cavity length (Fig. 14C: pt). Pallial vas deferens gradually becoming narrow, crosses to right corner of the pallial floor, as superficial, relatively narrow, protruded furrow, surrounding columellar muscle by a distance equivalent to 1/4 of pallial cavity length; abruptly turning left on pallial floor and entering the base of the penis. Penis long, somewhat flattened; proximal half of

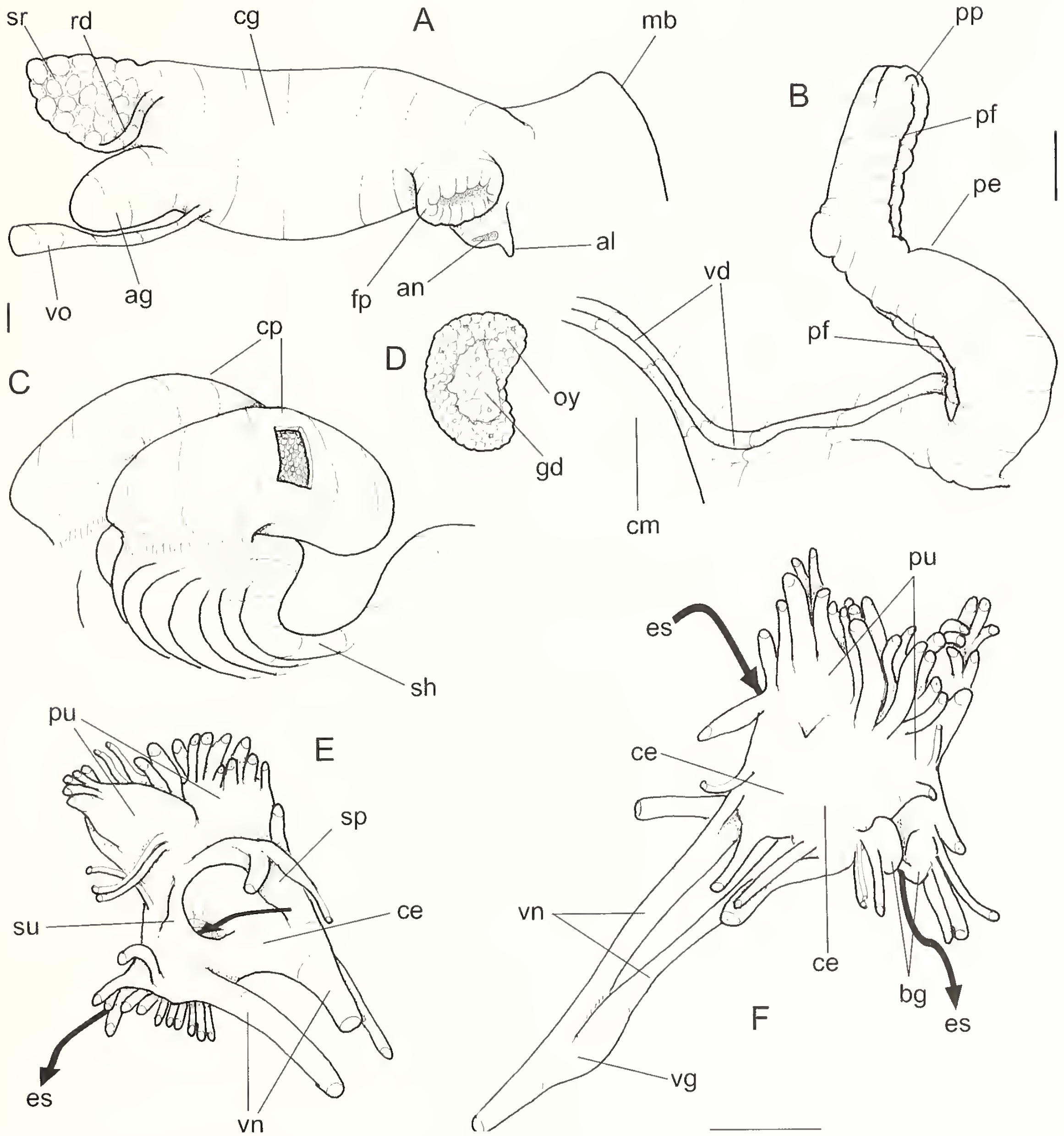

Figure 17. *Melapium lineatum* anatomy. A, pallial oviduct and some adjacent structures, ventral view. B, penis and adjacent region of its base, dorsal view, penis reflected upward. C, anterior region of shell, anterior view, partially showing the siphonal canal and a pair of egg capsules, with rectangular hole cut. D, visceral mass, female, transverse section through middle region of the penultimate whorl. E, central nervous system, ventral, right oblique view. F, same, dorsal, left oblique view, visceral ganglion (vg) included. Scale bars = 1 mm. Abbreviations listed in section with figure captions.

uniform width, with furrow running along its posterior edge; middle region with an irregular surface, furrow crossing to opposite penis edge; distal half about ⅔ as wide as proximal half, with furrow along its anterior edge; penis tip blunt, rounded, furrow leading to a small sub-terminal papilla (Fig. 17B).

Female (Fig. 17A). Ovary occupies entire surface of visceral whorls, covering entirely digestive gland, is thinner along the columellar surface (Fig. 17D). Visceral oviduct very narrow, running along middle ventral surface of visceral mass, running along the left edge of the kidney before entering the left edge of the pallial oviduct, between its posterior and middle thirds. Pallial oviduct with about same length of pallial cavity, protruding into the anterior region of the kidney and separated from mantle border by a distance equivalent to ¼ of pallial cavity length. The albumen gland, a small whitish appendix, comprises about 1/6 the size of the pallial oviduct and is located in the left half of the posterior pallial oviduct, ventral to the kidney, joining the capsule gland through a wide anterior opening. The seminal receptacle is equal in size to the albumen gland, and located alongside it, consisting of small, rounded lobes, several very narrow, iridescent ducts that run from posterior to anterior along left its edge and connect to the posterior end of the capsule gland. The capsule gland occupies most of the pallial oviduct, is yellowish and somewhat flattened dorso-ventrally, consisting of a pair of thick glandular lamellae. The female pore is sub-terminal, situated to the left of the anterior-most portion of the capsule gland at the end of a tall, wide, terminal papilla. Terminal papilla is situated slightly posterior and to the right of the anus, and is preceded by a small, hollow, thick-walled chamber continuous with the capsule gland lumen. There are no signs of a cement gland in sole of the foot.

Central nervous system (Figs. 17E-F). The nerve ring is highly concentrated, and it is difficult to distinguish the ganglia and connectives. Its total volume occupies roughly 1/10 of the haemocoel. The pedal ganglia are equivalent in size to the cerebral-pleural ganglia. The passage of the esophagus is narrow. A pair of buccal ganglia are located close to the cerebral ganglia. Supra and sub-esophageal ganglia are also located close to the nerve ring. The visceral ganglion is located posteriorly at a distance equivalent to the nerve ring length.

Measurements (in mm). NMNH S6362: 24.7 by 22.7; NMNH 59733: 26.5 by 23.6; NMNH V1899: 20.9 by 22.3; NMNH V9979: 26.2 by 24.0.

Distribution. South Africa.

Habitat. Fine sand.

Material examined. SOUTH AFRICA. **Eastern Cape** (dredged R.V. Meiring Naudé); Transkei, off Mbotyi, 31°29′02″S 29°45′04″E, 48-50 m depth 3♀, NMNH V9978, (sta. F5, viii/1981); off East London, 33°06.2′S 27°52.4′E, 70 m depth, 1♂, NMNH V9979 (sta. XX34, 16/vii/1984). **Western Cape**; Agulhas Bank, E of Martha Point, 34°29.5′S 20°33.3′E, 28 m depth, 1♂, NMNH S6362 (NMDP CC13, 7/iv/1991), 34°29.5′S 20°32.9′E, 24-28 m depth, 1♂, NMNH 59733 (MMDP CC14, 7/iv/1991); South Cape, south of Cape Infanta, 35°38′S 20°50′E, 90 m depth, 1♂, NMNH V1899 (MMDP sta. A16590D, 30/ix/1994).

Discussion. The genus *Melapium* was removed from Pseudolividae by Kantor (1991) and Vermeij (1998), based on anatomical and conchological peculiarities. One of the more conspicuous differences is the lack of spiral groove at the outer lip and the prominent entrance of the siphonal canal. Kantor (1991) erected a new family, the Melapiidae, to include this genus. Vermeij (1998), on the other hand, argued that *Melapium* can be placed in the Strepturidae. As several authors still include this genus in the Pseudolividae (OBIS 2004), this assignment is tentatively maintained here.

This study was built upon the anatomical description of Kantor (1991), but expanded to include developmental data and the different strategy of carrying the egg capsules.

Family Nassariidae
Genus *Nassodonta* H. Adams, 1867
(Type species *Nassa insignis* H. Adams, 1867, by monotypy)

Nassodonta dorri (Watteblet, 1886)
(Figs. 3G-J, 4G-I, 18A-19D)

Canidia dorri Watteblet, 1886: 56-57 (pl. 4, fig.2).
Nassodonta dorri: Kantor and Kilburn 2001: 99-104 (figs. 1-21).

Description

Shell (Figs. 3G-H). Fusiform, up to 15 mm; color greenish beige, with some sparse, weak, pale brown chevrons on the body whorl. Walls very thick. Protoconch eroded. Spire of about 4 convex whorls; suture deep; last two spire whorls with strong axial nodular threads, gradually disappearing towards body whorl. Body whorl mostly smooth except for growth lines; ventral region with about 5 wide, uniformly spaced, spiral furrows along its anterior half, the inferior furrow widest, encircling the siphonal canal. These furrows continue onto the dorsal surface of body whorl. The posterior furrow is wider, producing a small projection on the outer lip. Aperture elliptical, peristome whitish. Inner lip smooth, thick, lacking callus. Outer lip thick, blunt, with 2 or 3 low teeth along its central area. Canal wide, short, simple. Other details in Kantor and Kilburn (2001: 101).

The following description is based on re-hydrated semi-mummified specimens.

Head-foot (Figs. 18A-B). Head-foot consists of 1½ conical whorls. Head not obvious and inlaid, marked only by presence of tentacles. Tentacles about ½ as long as the foot, the proximal ⅔ clearly broader than distal ⅓. Eyes are well developed, located at outer sides tentacle prior to narrower region. The broad optical nerve is easily seen by transparency, running along the center of each tentacle. Tentacle bases adjacent. Each tentacle located close to each other, running parallel like a socket. Rhynchostome very narrow, located just ventral to region between tentacle bases. Foot wide, ample, simple, occupying about ⅓ of shell volume when retracted. Retraction is umbrella-like, producing a ventral concavity. The anterior furrow of pedal glands is surrounded by thick edges that are restricted to the anterior border of the foot, weakly expanding laterally. Ventral mantle insertion far posterior to pedal edges. Columellar

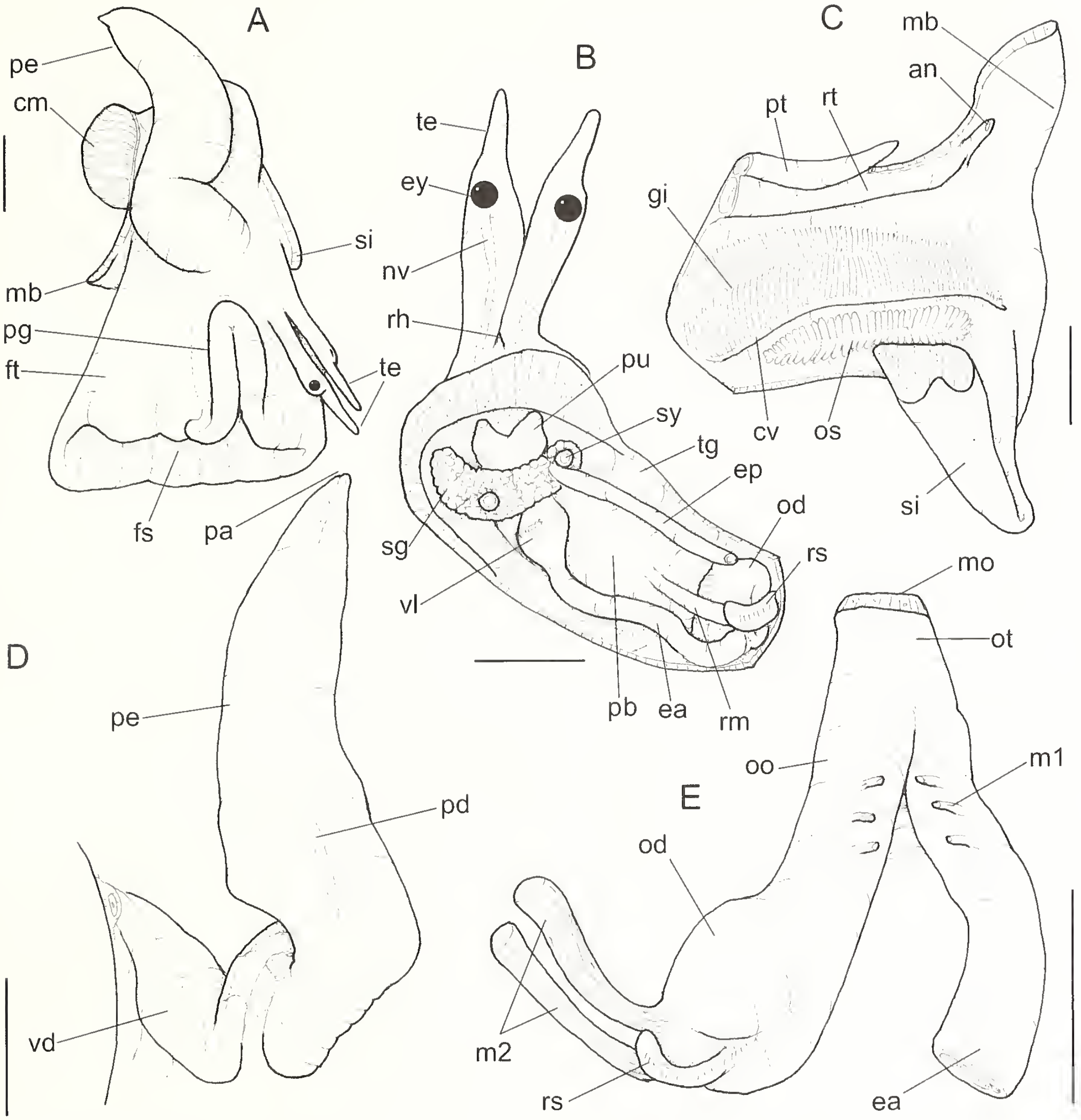

Figure 18. *Nassodonta dorri* anatomy. A, head-foot, male, frontal view. B, head and haemocoel, ventral view, foot and columellar muscle removed. C, pallial cavity, male, ventral-inner view. D, penis and adjacent region of its base, ventral view, penis partially reflected, portions of penis duct (pd) seen by transparency. E, buccal mass, left view. Scale bars = 1 mm. Abbreviations listed in section with figure captions.

muscle simple, of about ¾ whorl. Male with large penis inserted posterior to right tentacle, at short distance from median line. Haemocoel elliptical, relatively wide, oblique.

Operculum (Figs. 3I-J). Corneous, pale brown, elliptical, occupying entire aperture. Nucleus sub-terminal, anterior. Outer sculpture concentric, with weak spiral growth lines. Inner surface glossy; scar elliptical, deep, closer to inner edge, occupying about half of opercular area. Other details in Kantor and Kilburn (2001:101, fig. 14).

Mantle organs (Fig. 18C). Mantle border simple, somewhat thick, whitish. Siphon thick, short, conical, with simple edges, about ½ of pallial cavity length. Pallial cavity spans about 1 whorl. Osphradium long, elliptical, with an area equivalent to ⅓ of gill area, left filaments about half the size of the right filaments; anterior end rounded, located at base of siphon; posterior end pointed. Gill spans about ½ of pallial roof area, adjacent to the osphradium. Its anterior end is pointed, but broadens at first rapidly then gradually until the posterior ⅓ of the pallial cavity then narrowing gradually. Gill filaments are low, triangular, and have a central apex. The roof of the pallial cavity separates the gill from the rectum, which is narrow and runs along the right edge of the pallial cavity. The anus is simple, at the distal end of a short, detached portion of the rectum, at the level of the anterior end of the gill.

Visceral mass. Not seen.

Circulatory and excretory systems. Not seen.

Digestive system (Figs. 18B, 18E-19D). The proboscis is narrow, thin walled, about ¾ of the head-foot length (Fig. 18B: pb) with a thick muscular, sphincter-like base (Fig. 19A). Proboscis and buccal mass relatively short, with about ⅓ remaining in the retracted proboscis. Odontophore about ½ of proboscis length, about ½ protruding beyond the proboscis in retracted position. The oral tube with is about ½ the odontophore length and width, its inner surface smooth and simple. The odontophore and esophagus detach from each other just posterior to oral tube, but are bound to each other by a series of narrow lateral muscles (mt). Odontophore muscles (Figs. 18E-19D): **m1**, small muscles connecting lateral edge of odontophore tube with adjacent region of esophagus; **m2**, strong pair of buccal mass retractor muscles, originating from ventral surface of haemocoel, running anteriorly, mostly attached to ventral surface of the proboscis, inserting into the posterior end of the cartilages; **m2a**, pair of strong accessory buccal mass retractor muscles and accessory dorsal tensors of the radula (Figs. 19C-D), originating in same region as m2, running medially alongside the m2 pair, attached to the ventral surface of the proboscis, becoming broader after penetration into odontophore, inserting along lateral surfaces of radular sac; **m3**, superficial and thin pair of muscles, originating in dorsal region of m2a pair, in a region just anterior to their penetration into the odontophore, running along superficial membrane covering odontophore, splaying along anterior region of this membrane; **m4**, pair of strong dorsal tensor muscles of radula, originating in medial, dorsal, and posterior surfaces of cartilages, running anteriorly, inserting into the radular sac with m2a; **m4d**, narrow pair of accessory dorsal muscles of radula, having same parameters as m4 but running more dorsal, originating more dorsal and anterior to m4 origin, and inserting more medially, covering m4/m2a insertion; **m5**, pair of ventro-medial dorsal tensor muscles of the radula, originating on the outer-ventral surface of the posterior end of the cartilages (opposed to m4 and m4a origins), running anteriorly, covering the ventral surface of m4/m2a, inserting along the radular sac with m4/m2a; **m6**, thin, horizontal muscle, connecting both cartilages along their median-ventral edges, from a region just posterior to cartilages join, spanning roughly half the length of the odontophore, narrow anteriorly, gradually broadening posteriorly. **mj**, a pair of narrow odontophore protractor muscles, originating along the odontophore anterior tube (that connects odontophore to oral tube), gradually becoming thicker posteriorly, inserting in odontophore cartilages along the middle region of their dorsal edge; **mc**, circular muscle opposing m6, inserting along outer-dorsal edge of both cartilages (opposite to m6), surrounding dorsally all inner muscles (except m11a); **m11a**, pair of narrow ventral tensor muscles of the radula, originating on the median-dorsal region of the cartilages, just anterior to m4 origin, running anteriorly, covering mc, inserting into the ventral end of the subradular membrane (Fig. 19C). Subradular cartilage (**sc**) is a convex membrane covering the anterior region of the odontophore, protrudes into the buccal cavity (Fig. 19C). Odontophore cartilages (**oc**) are paired, long, narrow, flat, slightly broader anteriorly (Figs. 19B-C); their posterior ends rounded, narrow; fused along ventral edge along the anterior ¼ of their length. Radula about 1½ times odontophore length. Radular teeth (Figs. 4G-I): **Rachidian** arched, flat, spans ½ of radular ribbon width; with ~10 sharp, pointed cusps along the cutting edge, somewhat similar to each other, slightly shorter towards tooth margins, separated from each other by space equivalent to their size. Secondary, irregularly disposed, small cusps are sometimes present, mainly along the median region; lateral edge straight, ⅓ of rachidian width; posterior edge concave, well separated from neighboring tooth. **Lateral teeth** with a rectangular base about ⅔ as wide as the rachidian tooth, separated from rachidian tooth by a gap about 1/8 of the radular ribbon width. Lateral teeth are situated obliquely as if continuations of the rachidian tooth, each with ~5 broad cusps along its cutting edge. Cusps similar in size except for outermost cusp, which is about 3 times the length and slightly broader than remaining cusps. Outermost cusp curved inward. Innermost cusp with up to five very small secondary cusps along its inner edge. Other details of radula as reported in Kantor and Kilburn (2001: 101-102, figs. 15-20). **Salivary glands** are whitish, amorphous, and restricted to the anterior region of haemocoel, surrounding the proboscis base and nerve ring. Neither their ducts nor the accessory salivary glands were found (Fig. 18B: sg). The anterior esophagus (Fig. 18B: ea) is narrow, originating from the oral tube (ot) after a distance equivalent to half the length of the odontophore. 3-4 pairs of narrow, well-separated slender pairs of jugal muscles connect the lateral surface of anterior esophagus to the lateral surface of odontophore tube (Fig. 18E: m1). Inner surface of anterior esophagus smooth. Anterior esophagus length equivalent to that of the proboscis. Valve of Leiblein about ¼ odontophore volume in size, located just posterior to nerve ring, at the ventral base of the proboscis

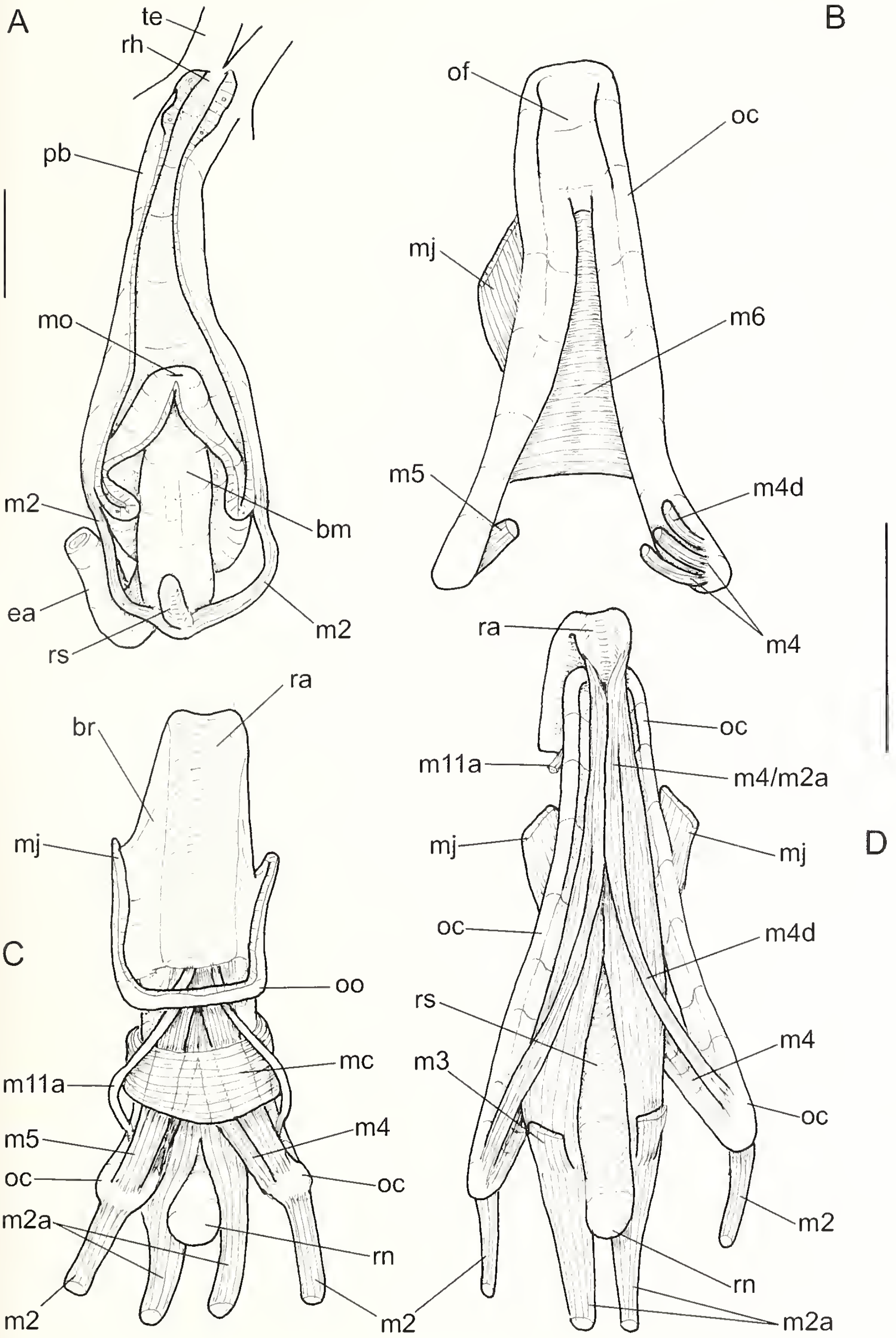
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Figure 19. *Nassodonta dorri* anatomy. A, foregut, ventral view, proboscis (pb) opened longitudinally, head region also shown. B, odontophore cartilages, dorsal view, insertion of some muscles also shown. C, odontophore, ventral view, superficial layer of membrane and muscles partially removed, structures of posterior region partially reflected. D, same, dorsal view, superficial layer of membrane and muscles removed, ventral portion of radula partially reflected forwards. Scale bars = 1 mm. Abbreviations listed in section with figure captions.

(Fig. 18B). the gland of Leiblein was not found. The posterior esophagus is narrow, running along the left side of the haemocoel. Midgut not examined. Rectum and anus are described above (pallial cavity).

Genital system. Male (Figs. 18A, 18D). Visceral structures not examined. Prostate gland narrow, closed (tubular), running ventral to and to the right of the rectum for 3/4 of its length (Fig. 18C: pt) before narrowing and abruptly crossing to the pallial floor. The pallial vas deferens is broadest just after descending to the pallial floor, narrows and forms a zigzag on the surface of the pallial floor leading to the penis base (Fig. 18D: vd). The penis is about half as long as the head-foot (Fig. 18A), its base twisted and oval in section. The penis widens somewhat abruptly at mid-length forming a blunt bulge along its right edge. The median and distal thirds of the penis are flat (Fig. 18D), gradually tapering to a pointed tip. The penis is duct narrow, running medially to the penis tip, where it opens.

Female. No well-preserved female examined. Visceral structures not seen. The pallial oviduct is whitish, closed (tubular), about twice the width or the rectum, and apparently lacks an anterior bursa copulatrix. The sole of the foot of females with a thick walled, whitish, glandular cement gland situated medially in the anterior half of the foot, as deep as half the foot width.

Central nervous system. Only pedal ganglia well-preserved, located in ventral region of proboscis base close to each other and to median line (Fig. 18B: pu). Each ganglion occupies a volume about 1/8 of the odotophore. Statocysts with large statolith, each located in the ventral haemocoel surface; partly immersed in the salivary glands and partly in the local pedal musculature.

Measurements of shells (in mm). MZSP 53533: ♂1: 13.6 by 8.6; ♂2: 12.0 by 7.7; ♂3: 12.0 by 8.0; ♀4: 14.2 by 8.3.

Distribution. Vietnam.

Habitat. Brackish, practically freshwater muddy environment.

Material examined. VIETNAM; Kolan, MZSP 53533, 2 shells, 3♂, 2♀ re-hydrated soft parts, Gemert private collection, 3 shells (beach of river).

Discussion. The shell of *Nassodonta* is easily confused with that of pseudolivids, being virtually identical to that of the genus *Macron* H. and A. Adams, 1853. It differs conchologically mainly in having a shorter spire. As discussed by Vermeij (1998: 70-71), *Macron* was referred to the Nassariidae. Based on its anatomy, *Nassodonta* undoubtedly belongs to the Nassariidae–Buccinidae, mainly on the basis of odontophore features.

CHARACTERS

Shell

1. Shell spiral furrow at last whorl: 0 = absent; 1 = present (*Zemira, Fulmentum, Benthobia, Nassodonta*) (CI = 50; RI = 50).

   The spiral furrow dividing the shell body whorl traces the position of the labral tooth on the outer lip, although this tooth is not well-developed in all species. It is normally present as a shallow, oblique furrow between the middle and anterior thirds of the body whorl. This furrow is one of the more conspicuous pseudolivid shell features, and has been called as "pseudolivid groove" (Vermeij 1998).

   However, some taxa considered to be pseudolivids, such as *Melapium*, lack this furrow, while a furrow and labral tooth is present in other genera that unquestionably belong to other families. Some examples include: *Acanthina* Waldheim, 1807 (Muricidae); *Leucozonia* Gray, 1847 (Fasciolariidae); *Ancilla* Lamarck, 1799 (Olividae); and *Bivetiella* Wenz, 1938 (Cancellariidae). These indicate the high degree of convergence in this character.

2. Tooth on outer lip: 0 = absent; 1 = present (*Zemira, Fulmentum*) (CI = 50; RI = 0).

   Although the furrow normally is associated with a tooth at the outer lip, also called labral tooth, this character only refers to a well-developed one, and not to a small projection. Apparently, the tooth at the outer lip is associated with predation on bivalves, serving to separate the valves. In the present study, it is equally parsimonious to consider state 1 as supporting node 4 reverting in node 6 or as a convergence between *Zemira* and *Fulmentum*, the first hypothesis is shown in the Fig. 1.

3. Determinate growth: 0 = present; 1 = practically absent (*Melapium, Fulmentum, Nassodonta, Benthobia*) (CI = 33; RI = 0).

   Determinate growth is the development of a differentiated peristome when the animal becomes mature. This feature is well explored by Vermeij and Signor (1992) and is here applied. As determinate growth is present in most of higher Caenogastropods (Simone 2000), it is considered plesiomorphic for neogastropods, and its absence, *i.e.*, the non-determinate growth is here considered a derived reversal. As some species considered in state 1 have a weak thickness of the outer lip, the word "practically" is introduced.

Head-foot

4. Cephalic tentacles: 0 = separated (*Melapium, Benthobia*); 1 = joined to each other (*Nassodonta, Fulmentum, Zemira, Siratus*) (CI = 50; RI = 66).

   The cephalic tentacles placed together, close to the median line. This kind of modification is different from the normal feature in higher caenogastropods, which possess the state 0.

5. Foot posterior furrow: 0 = absent; 1 = present (*Benthobia*) (CI = 100; RI = 100).

   The conspicuous posterior furrow on the sole of the foot is restrict to the genus *Benthobia* and may be a character of the genus. It is discussed in Simone (2003).

6. Columellar muscle: 0 = simple; 1 = double (with a siphonal branch) (*Siratus, Melapium*) (CI = 100; RI = 100).

   The posterior region of the columellar muscle is normally a simple and broad flap. However, in the species listed under state 1, this region is bifid, having a wider left branch and another slender and taller right branch (Figs. 14A-B: cm). This feature has been commonly found in muricids according to my experience, and is related to an anterior furrow internally on each whorl, maintained by the siphonal canal, into which the right branch fits.

7. Operculum nucleus: 0 = terminal (*Fulmentum, Benthobia*); 1 = sub-terminal (*Nassodonta, Zemira*); 2 = almost central (*Siratus*); 3 = absent (*Melapium*) (CI = 75; RI = 0; not additive).

   An operculum with a terminal nucleus is a modified condition in gastropods, but among the neogastropods, this is the plesiomorphic condition. The remaining states, including the loss of the operculum, are considered further modifications. While the loss of the operculum is certainly a different phenomenon from the position of the nucleus, these are joined because of the non-additive condition. This is mathematically equivalent to considering the loss as a separate character. With respect to the state allocation in the cladogram, it is equally parsimonious to consider state 1 as supporting node 3, then reversing in *Fulmentum*, or a convergence between *Nassodonta* and *Zemira*. The first hypothesis is shown in the Fig. 1.

Pallial organs

8. Siphon: 0 = long; 1 = short, almost inconspicuous (*Benthobia, Zemira*) (CI = 50; RI = 50).

   The siphon is a modification of the mantle border and is distinct from the development of a siphon in the shell. There are taxa that possess a siphon in the shell, yet lack a developed siphon in the mantle, as, *e.g.*, Stromboidea (Simone 2005) and Cerithioidea (Simone 2001), while other taxa possess a well-developed siphon at mantle border, but lack any special modification in the shell, as, *e.g.*, Calyptraeoidea (Simone 2002). The long and exploratory pallial siphon is the rule in Hypsogastropoda, and is considered to be plesiomorphic in neogastropods. State 1 is considered a reduction.

9. Osphradium length relative to gill length: 0 = shorter than half (*Siratus*); 1 = longer than half (*Melapium, Fulmentum, Nassodonta*); 2 = almost same length (*Zemira, Benthobia*) (CI = 50; RI = 33; additive).

   Although the states are optimized as additive, based on ontogeny, identical results and indices are produced when the character is considered not additive.

10. Osphradium: 0 = symmetrical; 1 = asymmetrical (*Benthobia, Nassodonta*) (CI = 50; R I= 50).

    Symmetry refers to the left and right filaments being symmetrical about the axis of the osphradial gangilion, with the filaments of both sides similar sized. It is equally parsimonious to consider state 1 as convergent between node 2 and *Nassodonta*, or supporting node 1 and reverting in node 4; the first hypothesis is shown in the Fig. 1.

11. Osphradium with monopectinate anterior portion: 0 = absent; 1 = present (*Benthobia*) (CI = 100; RI = 100).

    Osphradium characters (9-11) are normally connected to reduction in body size. The smaller the animal, the larger, proportionally, is the osphradium. The same can be concluded with regard to the asymmetry of the osphradium filaments. Smaller animals tend to have the left filaments smaller than the right ones. The loss of the left filaments (character 11) can be considered as the extreme of this tendency to miniaturization. In the case of *Benthobia*, the monopectinate condition is only present in the anterior portion of the osphradium (Simone 2003: figs. 7B, 9B, 11A, 12A).

Digestive system

12. Ventral chitinous platform within the oral tube: 0 = absent; 1 = present (*Melapium, Zemira, Siratus, Fulmentum*) (CI = 100; RI = 100).

    The chitinous platform is a relatively thick longitudinal band located along the ventral surface of the oral tube. It starts close to the point where the odontophore enters the oral tube, and ends close to the mouth. This platform is particularly well developed in muricids that I have examined, but it is also present in the above listed other species (Figs. 7F, 15E: ol). The function of this structure is unknown, but normally the salivary glands open in the middle level of its lateral edges, which suggests a relationship between the glands and the chitinous platform. This reinforcement of the inner surface of the oral tube may possibly be linked to contact with the radular teeth, serving to avoid self-injury. There does not appear to be a direct relationship with the jaws, which are located on the dorsal surface of the oral tube, and are normally absent in neogastropods.

13. Length of odontophore horizontal muscle (m6): 0 = about half the length of the cartilages; 1 = almost

the same length as the cartilages (*Nassodonta, Fulmentum, Melapium, Zemira, Siratus*) (CI = 100; RI = 100).

The horizontal muscle (m6) connects both odontophore cartilages to each other along their ventral edge. In neogastropods, however, this muscle is normally thin and tends to become longer, almost as long as the cartilages. This feature is explored in this character (Figs. 6B, 12A, 16C-D, 19B).

14. Jaw muscle (mj): 0 = thin, as a flap; 1 = as a separate band (*Siratus, Zemira, Melapium, Nassodonta*) (CI = 50; RI = 66).

The jaw muscles (mj) are modified in neogastropods because of the greater development of the odontophore tube, which makes the connection between it and the oral tube. This modification may be responsible for the further alteration of state 1. Although the neogastropods normally lack jaws, as is the case in the species examined, this name is here maintained in order to indicate the homology of this structure with those of the remaining caenogastropods.

15. Odontophore ventral tensor muscle of radula (m11a-m4v): 0 = absent; 1 = present (all taxa in this study) (CI = 100; RI = 100).
16. Dorsal tensor muscles of radula m4 and m5: 0 = separated from each other; 1 = continuous with each other (*Siratus, Fulmentum, Melapium, Zemira, Nassodonta*) (CI = 100; RI = 100).
17. Connection of m4 with inner surface of cartilages: 0 = absent; 1 = present (*Siratus, Melapium, Fulmentum*) (CI = 100; RI = 100).
18. Odontophore cartilage outline: 0 = elliptical; 1 = elongated (*Siratus, Melapium, Nassodonta*) (CI = 50; RI = 50).
19. The odontophore of the muricoideans (broad sense) is different from those of the remaining caenogastropods in two main features. The first is the tendency to elongation, which results in odontophores that may be as long as the proboscis. They extend into the haemocoel when the proboscis is retracted. Another difference is the development of ventral tensor muscles of the radula. This muscle pair is present in archaeogastropods, but is practically lost in caenogastropods. The muricoideans revert to this condition, re-acquiring the ventral tensor muscles from a modification of the dorsal ones. The modifications resulting from these tendencies are explored in above characters (15-18). Odontophore tube connecting odontophore to the oral tube: 0 = absent or very short; 1 = long (all taxa in this study) (CI = 100; RI = 100).

The odontophore tube is a separate structure from the well-known "oral tube" (Simone 2003, fig. 7G: oo) (Figs. 15D, 18E: oo). This muscular tube connects the odontophore to the oral tube. Elongation of this tube is another common character of the muricoideans, in which the buccal mass structures become V-shaped, with the mouth at the vertex of this "V".

20. Esophageal origin: 0 = posterior to odontophore; 1 = anterior-dorsal to odontophore (buccal mass V-shaped) (all taxa in this study) (CI = 100; RI = 100).

In most caenogastropods, the buccal mass and esophagus are linear, *i.e.*, the origin of the esophagus is in the posterior region of the odontophore. As noted above, the buccal mass and esophagus may be V-shaped in the muricoideans, with the anterior esophagus and the elongate odontophore running parallel to each other.

21. Accessory salivary gland: 0 = absent (*Zemira*); 1 = paired (*Siratus, Melapium?*); 2 = unpaired (*Fulmentum, Benthobia*) (? = *Nassodonta*) (CI = 66; RI = 50; not additive).

As the accessory salivary gland has been considered as a synapomorphy of the Neogastropoda (Ponder 1974, Haszprunar 1988), its absence is considered plesiomorphic. This condition in *Zemira* is most likely a reversion. Kantor (1991) had considered, however, two apomorphic states of the accessory salivary gland, absent and the single (unpaired) condition.

The presence (state 2) in *Fulmentum* can be considered an autapomorphy for this taxon (convergent with node 2) or supporting node 5. The second hypothesis is shown in the Fig. 1, but in this hypothesis the pair of accessory salivary gland originated from a single gland.

22. Valve of Leiblein: 0 = absent; 1 = present (all taxa in this study) (CI = 100; RI = 100).
23. Valve of Leiblein oblique furrow: 0 = absent; 1 = present (*Zemira, Melapium, Fulmentum, Nassodonta, Siratus*) (CI = 100; RI = 100).

The valve of Leiblein is considered to be another synapomorphy of the Neogastropoda (Haszprunar 1988), and is certainly present in most muricoideans. As nothing similar can currently be attributed to the Conoidea, the presence of this valve is here considered to be an apomorphic state (character 22), supporting a branch uniting muricoideans and cancellarioideans. Its internal organization, on the other hand, has not been studied in detail. Comparisons at this level are very difficult, as information is inadequate. Certainly, the valve of Leiblein is a complex structure, the function of which is uncertain. In some species, the valve of Leiblein has a transverse furrow that can be considered as homologous to the bypass shown by Ponder (1974: fig. 3), a way for the food pass directly to the middle esophagus without passing through the valve. This condition is considered apomorphic (Figs. 7B, 12B, 15C), being one of the synapomorphies of the node 3.

24. Gland of Leiblein: 0 = absent; 1 = present (all); 2 = elongated (*Zemira, Melapium*) (CI = 66; RI = 0; additive).

    The gland of Leiblein is another synapomorphy of the Neogastropoda (Ponder 1974; Haszprunar 1988). It is further modified in several taxa, including its disappearance and its modification into a venom gland in the conoideans. The modification explored here is the elongated form. In this pattern, the gland is stored inside the haemocoel intensely coiled. It is shown in Figs. 7C and 15B artificially uncoiled. The additive condition is based on ontogeny, as very young specimens possess a shorter gland, however, nothing changes in the result or indices if the character is considered not additive.

25. Gland of Leiblein duct: 0 = short; 1 = long (about half the length of the middle esophagus) (*Siratus, Zemira, Melapium, Fulmentum*) (CI = 100; RI = 100).
26. Gland of Leiblein duct: 0 = with transversal septa (*Siratus*); 1 = glandular (*Zemira, Fulmentum*); 2 = simple (*Melapium, Benthobia, Nassodonta*) (CI = 40; RI = 25; additive).

    The gland of Leiblein characters (24-26) are based on the hypothesis that this gland is a modification of the middle esophageal gland, present in the higher mesogastropods (Naticoidea, Cypraeoidea, Tonnoidea). In these taxa, the esophageal gland consists of a series of transverse septa. Something similar is also found in some muricoideans, but reduced and located in the duct of gland of Leiblein, further supporting the link between these two structures. The presence of such septa in the gland duct is considered plesiomorphic. The glandular condition of the duct is considered as an intermediate step for a simple-duct condition. This is the reason for considering the states in an additive optimization; however, if considered not additive, the resulted cladogram is the same, but the indices change to CI = 50 and RI = 0.

27. Stomach form: 0 = a simple curve; 1 = with a dilated chamber posterior to esophageal insertion (*Fulmentum, Melapium, Zemira, Siratus*) (CI = 100; RI = 100).
28. Number of stomach ducts to the digestive glands: 0 = 2 (*Melapium*); 1 = 1 (*Benthobia, Fulmentum, Zemira, Siratus*) (?= *Nassodonta*) (CI = 50; RI = 0).

    The stomach of normally carnivorous neogastropods, is, in most taxa, a simple curve. However, some taxa have developed a more complex stomach that is considered to be apomorphic herein. The number of the ducts leading to the digestive gland tends to be simplified, form a pair, as normal in lower caenogastropods, to a single duct.

29. Anal papilla: 0 = absent; 1 = present (*Siratus, Melapium, Fulmentum*) (CI = 100; RI = 100).

    The anal papilla is not a conspicuous structure, but is present along the dorsal margin of the anus in above mentioned species (Figs. 10A, 14C: al), as well as in the remaining muricids examined. It most likely represents a synapomorphy.

Genital system

30. Penis duct: 0 = open (a furrow) (*Siratus, Melapium, Fulmentum*); 1 = closed (a tube) (*Zemira, Nassodonta, Benthobia*) (CI = 50; RI = 66).
31. Penis retractile terminal broad papilla: 0 = absent; 1 = present (*Benthobia, Fulmentum*) (CI = 50; RI = 50).

    The male genital system is not normally well preserved, as most of the visceral structures are lost or not extracted without damage. This is the case of the present sample. However, comparisons were made among those species for which material was available. The opened condition (character 30) is considered plesiomorphic, based on the condition found in most basal caenogastropods, but the closed (tubular) condition commonly occurs throughout the caenogastropods as convergences.

32. Bursa copulatrix: 0 = a blind sac; 1 = terminal, as continuation of oviduct (*Benthobia, Fulmentum, Zemira*); 2 = absent (*Siratus*) (not additive) (CI = 66; RI = 0).

    The polarization of this character is also based on the condition normally found in other caenogastropods, mainly higher mesogastropods. Although several interesting differences in the female genital system were found, all appeared to be autapomorphic, except for the condition of the bursa copulatrix.

Central nervous system

33. Nerve ring ganglia: 0 = ganglia separated; 1 = ganglia almost fused (all) (CI = 100; RI = 100).
34. Buccal ganglia: 0 = close to buccal mass; 1 = close to nerve ring (all) (CI = 100; RI = 100).

Both central nervous system characters are polarized based on the remaining caenogastropods, in such the ganglia are clearly separated from each other (character 33), and the paired buccal ganglia are located far from the nerve ring and closer to the buccal mass (34). Both conditions are further modified in the above mentioned taxa.

TAXONOMY

The cladogram based on the set of characters shown in the Table 1 is depicted in figures 1 and 2. Character polarity is based mainly on Tonnoideans, *i.e., Tonna galea* (Linné, 1758) and *T. maculosa* (Dillwyn, 1817) (Simone 1995), as well as other species still under study. Based on this scenario, the Conoidea share seven synapomorphies with the ingroup, the more important being determinate shell growth (character 3), the presence of the odontophore ventral tensor muscle of radula (15), the gland of Leiblein (character 24), closure of penis duct (character 30), and the adaptations of the nerve ring (characters 33, 34).

The ingroup also is supported by eight synapomorphies (**node 1**), the more important being the spiral furrow in the last shell whorl (character 1), the elongation of the osphradium (9), the odontophore tube (19), the valve of Leiblein (22), the further modification of the gland of Leiblein duct (26), and the reduction of the stomach ducts (28).

Node 2 represents the genus *Benthobia*, based on data from Simone (2003). Although five species had been studied, only two are included here because of the completeness of data. This node is supported by 9 synapomorphies, two are non-homoplastic: the posterior furrow of the foot (character 5) and the monopectinate condition of the anterior region of the osphradium (11). The features are convergent with other branches of the cladogram. Among the more notable are: the shortness of the siphon (8), the osphradium equal in length to the gill (9), a single accessory salivary gland (21) and the terminal papilla of the penis.

Node 3, the remaining ingroup, is supported by six synapomorphies, the more conspicuous are: the close situation of the cephalic tentacles (character 4), the sub-terminal condition of the opercular nucleus (7), the length of the horizontal muscle of odontophore (13), and the oblique furrow of the valve of Leiblein (23).

The next dichotomy separates the nassariid *Nassodonta dorri* from the remaining ingroup taxa (**node 4**). The taxonomy of the genus *Nassodonta* has been analyzed by Kantor and Kilburn (2001), who provided a history and additional comments.

However, there is a remarkable similarity in shell characters with the pseudolivid taxa. As pointed by Kantor and Kilburn (2001: fig. 13), *Nassodonta* also possesses a deep basal spiral furrow in the anterior region of body whorl, mostly associated with a labral tooth. On the other hand, the anatomical characters such as the radula and the degree of fusion between both odontophore cartilages clearly show the nassariid nature of the *Nassodonta*.

Node 4 is supported by five synapomorphies, the more interesting being: the tooth at outer shell lip (character 2) that reverts in the node 6, the ventral chitinous platform in the oral tube (12), further elongation of the duct of the gland of Leiblein (25) and the dilatation of the stomach (27).

Node 5 unites the remaining ingroup species except *Zemira australis*, and is supported by four synapomorphies, most noteworthy being the single accessory salivary gland (character 21) and the anal papilla (29).

Node 6 is supported by five synapomorphies, and unites *Melapium*, that mostly is considered a pseudolivid, with the muricid *Siratus senegalensis*. Of the synapomorphies for this node, the more important are the double condition of the columellar muscle (character 6) and the paired state of the accessory salivary glands.

Based on this scenario, the formal family Pseudolividae, in the present sense, is not monophyletic. Four of the included genera (*Benthobia, Zemira, Fulmentum*, and *Melapium*) are mixed with a nassariid (*Nassodonta*) and a muricid (*Siratus*). Although the studied set of species represents only a subset of the genera included in the Pseudolividae, it appears to be is sufficient to demonstrate the polyphyletic nature of the taxon. Since the type species of *Pseudoliva* Swainson, 1840, the type genus of the family Pseudolividae, *P. crassa* (Gmelin, 1791), was not studied, no definite conclusions about the taxonomy of Pseudolividae can yet be reached. However, at least the present concept of the family level taxon has been shown as lacking phylogenetic support.

Among the species studied, *Fulmentum ancilla* is closest to *Pseudoliva*. Some authors consider this species to belong to *Pseudoliva* (*e.g.*, Kantor 1991, Hayes 1994). If *P. crassa* is close to *F. ancilla*, the cladogram indicates that it, and with *Melapium*, could be considered as belonging to Muricidae or, at least, a sister taxon of that family. According to this tree topology, the genera *Zemira* and *Benthobia* could be placed in other families. Kantor (1991: 34) provided some anatomical information on *Pseudoliva zebrina* A. Adams, 1853, which shares similarities with *F. ancilla*, mainly with respect to foregut characters. This can further indicate a close relationship between *Fulmentum* and *Pseudoliva*.

The phylogenetic analysis by Kantor (1991: fig. 19) shows four synapomorphies supporting the monophyly of the Pseudolividae (except *Melapium*). Three of the synapomorphies (three teeth per radular row, a short free portion of the duct of salivary gland, and the anal gland) occur commonly in the Muricoidea. A fourth synapomorphy (accessory esophageal gland) is a distinctive character, but the variability of form and position of this gland, and its presence in several other muricoideans, suggest the possibility of convergence. While these four anatomical characters are the basis for recognizing the family Pseudolividae, none of them emerged as important synapomorphies in the present study. Similar arguments can be made with regard to the seven synapomorphies proposed for *Melapium* in that paper.

The outcome of the present analysis shows Pseudolividae, as currently understood, to be polyphyletic, contradicting previously published results, and indicating that the present concept of this taxon must be reevaluated. Most probably some of the genera now assigned to Pseudolividae will be found to belong to other families, while the name Pseudolividae is apparently only applicable only to the genera *Pseudoliva* and *Fulmentum*.

The definitions and limits of the families of the muricoideans only can be refined after a much wider analysis, including samples of much more representatives.

CONCLUSIONS

(1) The family Pseudolividae, in the present concept, is polyphyletic and must be not used as a formal taxon.

(2) Detailed morphology is valuable for comparative studies, as all examined species differ greatly in most structures.

(3) The genera *Zemira*, *Fulmentum*, and *Melapium* share synapomorphies with the genus *Siratus* (Muricidae). These taxa also share further synapomorphies with the *Nassodonta* (Nassariidae), being separated by them from *Benthobia*.

(4) No special taxonomical re-arrangement is proposed because of weakness of definition of the Muricoidea families.

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FIGURE CAPTIONS

In the figures, the following abbreviations are used: **aa**, anterior aorta; **ac**, auricle connection with kidney chamber; **ad**, accessory salivary gland duct; **ae**, accessory salivary gland; **af**, afferent gill vessel; **ag**, albumen gland; **al**, anal papilla; **an**, anus; **ao**, posterior aorta; **at**, vaginal atrium; **au**, auricle; **ba**, bursa copulatrix aperture; **bc**, bursa copulatrix; **bg**, buccal ganglion; **bm**, buccal mass; **br**, subradular membrane; **ce**, cerebral-pleural ganglia; **cg**, capsule gland; **cm**, columellar muscle; **cp**, capsule; **cv**, ctenidial vein; **dc**, dorsal chamber of buccal mass; **dd**, duct to digestive gland; **df**, dorsal fold of buccal mass; **dg**, digestive gland; **di**, diaphragm membrane separating haemocoel from visceral cavity; **ea**, anterior esophagus; **ef**, esophageal folds; **em**, middle esophagus; **ep**, posterior esophagus; **es**, esophagus; **ey**, eye; **fp**, female pore; **fs**, foot sole; **ft**, foot; **gi**, gill or gill filament; **gl**, gland of Leiblein; **gm**, gill muscle; **gp**, pedal ganglion; **he**, head; **hg**, hypobranchial gland; **in**, intestine; **ir**, insertion of m4 in tissue on radula (to); **is**, insertion of m5 in subradular membrane; **kc**, membrane between kidney and pericardium; **kd**, dorsal chamber of kidney; **ki**, kidney; **kl**, kidney dorsal lobe; **km**, membrane between kidney and pallial cavity; **kv**, ventral lobe of kidney; **ld**, duct of gland of Leiblein; **lg**, secondary gland of duct of gland of Leiblein; **m1** to **m14**, extrinsic and intrinsic odontophore muscles; **mb**, mantle border; **mc**, circular muscles of odontophore; **mf**, muscle fibers; **mj**, jaws, buccal, and oral tube muscles; **mo**, mouth; **ne**, nephropore; **ng**, nephridial gland; **nr**, nerve ring; **nv**, nerve; **oa**, opercular pad; **oc**, odontophore cartilage; **od**, odontophore; **of**, odontophore cartilage fusion; **oi**, opercular insertion; **ol**, oral tube ventral chitinous platform; **oo**, odontophore tube connecting to oral tube; **op**, operculum; **os**, osphradium; **ot**, oral tube; **oy**, ovary; **pa**, penis aperture; **pb**, proboscis; **pc**, pericardium; **pd**, penis duct; **pe**, penis; **pf**, penis furrow; **pg**, pedal glands anterior furrow; **pp**, penis papilla; **pt**, prostate; **pu**, pedal ganglion; **py**, pallial cavity; **ra**, radula; **rd**, seminal receptacle duct; **rh**, rhynchostome; **rm**, retractor muscle of proboscis; **rn**, radular nucleus; **rs**, radular sac; **rt**, rectum; **rv**, renal efferent vessel; **sa**, salivary gland aperture at oral tube; **sc**, subradular cartilage; **sd**, salivary duct; **se**, septum between esophagus and odontophore in buccal mass; **sg**, salivary gland; **sh**, shell siphon canal; **si**, siphon or siphon insertion; **sp**, supra-esophageal ganglion; **sr**, seminal receptacle; **st**, stomach; **su**, subesophageal ganglion; **sv**, seminal vesicle; **sy**, statocyst; **te** cephalic tentacle; **tg**, integument; **to**, tissue on middle region of radula preceding buccal cavity; **ts**, testis; **va**, vaginal duct; **vd**, vas deferens; **ve**, ventricle; **vf**, oblique furrow of valve of Leiblein; **vg**, visceral ganglion; **vn**, visceral nerve; **vl**, valve of Leiblein; **vo**, visceral oviduct.

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Gastropod mating systems: An introduction to the symposium*

Janet L. Leonard

Joseph M. Long Marine Laboratory, University of California-Santa Cruz, Santa Cruz, California 95060, U.S.A., jlleonar@ucsc.edu

Key words: reproduction, sexual selection, sexual behavior

Sex is what organisms are all about, and gastropods are no exception. The sexual behavior and reproductive biology of gastropods has fascinated naturalists from earliest times. The aerial mating behavior of *Limax maximus* Linnaeus, 1758, the chains of copulating *Aplysia* Linnaeus, 1767, the love dart of *Helix* (Linnaeus, 1758), the egg cases of whelks and naticids, and the delicate gelatinous egg masses of nudibranchs have been objects of wonder and speculation for centuries and the more we learn about such phenomena, the more marvelous they seem. Our childish pleasure at the delicacy and symmetry of a moon snail's egg collar is only enhanced by the understanding of its importance in allowing eggs to develop on muddy substrata; our astonishment at the length of the entwined penes that suspend a pair of mating *L. maximus* is only intensified by consideration of the conflicting pressures of natural and sexual selection that must have produced the phenomenon. The papers in this volume provide a wealth of new pleasures both by describing new and fascinating observations in gastropod sexual biology and by providing deeper insights into some of the more familiar systems.

The term mating system is shorthand for the species-typical reproductive behavior of a species: that is, who mates, when they mate, who is successful and why. The mating system is a product of both natural and sexual selection and is, in a sense, the grand finale to the life history of a species. Understanding the mating system of a species requires knowledge of many aspects of its ecology, physiology, and behavior. There is perhaps no species, including our own, for which the mating system is completely understood. However, in recent decades tremendous progress has been made in understanding mating systems from the standpoint of behavioral and evolutionary ecology. Modern mating systems theory views the mating system as the outcome of selection acting on selfish individuals who may have conflicting interests, even as they come together to produce and perhaps, rear, their offspring. Much of this work has dealt with humdrum and boring taxa such as birds, mammals, and insects but application of what Eric Charnov (1982) has termed, "selection thinking" to invertebrates, including gastropods, came early, with the publication of Mike Ghiselin's (1974) book, "The Economy of Nature and the Evolution of Sex" and George Williams's (1975) "Sex and Evolution". However, it has taken time for malacologists to embrace sexual selection and mating systems theory for a variety of reasons; many gastropods are hermaphrodites and the application of sexual selection theory to hermaphrodites is not entirely straightforward (see review in Leonard 2006); Darwin's idea that gastropods lacked the sensory and mental capacity to choose mates has been very influential and has had much intuitive appeal. However, it has been shown that the hermaphroditic basommatophoran, *Bulinus truncatus* (Audouin, 1826) can discriminate among mates based on their infection status and that this differs with the genotype of the chooser (Webster and Gower, 2006).

Over the last three decades, gradually and one by one, a variety of laboratories have begun to explore the sexual biology of gastropods as models for testing predictions of mating system theory and to use mating systems theory to understand the biology of the gastropods they are interested in. The immediate stimulus for the current symposium came from the realization that a certain critical mass has been reached and that it was time to bring together a selection of these workers from around the world to compare notes and provide an overview into the diversity of gastropod biology and gastropod research. The joint meeting at Asilomar seemed to be the ideal opportunity and the resulting symposium, "Gastropod Mating Systems" on the morning of June 27, 2005 consisted of nine talks, covering a wide variety of topics from the genetics of sex ratio (Yusa, this volume), to reproductive physiology (Ter Maat *et al.* and Mayeri) and paternal care (Grosberg). Three of the talks dealt with prosobranchs; two with opisthobranchs and four with pulmonates; one with the basommatophoran, *Lymnaea stagnalis* (Linnaeus, 1758), and three with stylommatophorans. Two of the talks are unfortunately not represented in this volume:

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* From the symposium "Gastropod Mating Systems" presented at the joint meeting of the American Malacological Society and Western Society of Malacologists, held 26-30 June 2005 at Asilomar, Pacific Grove, California.

> "Mating systems and family conflicts in a marine snail" by Rick Grosberg, Center for Population Biology, University of California-Davis

and

> "Mating and egg-laying behavior in *Aplysia* – Pheromones and neural mechanisms" by Earl Mayeri, Department of Physiology, University of California-San Francisco

However, we have added two important papers from authors who were not able to attend the symposium: a review of the mating system and reproductive biology of *Arianta arbustorum* (Linnaeus, 1758) by Bruno Baur of the University of Basel and a review of work on dart-shooting in helicids by Ronald Chase of McGill University. Several of the papers (by Yusa, Reise, Davison, Baur, and Chase) represent important reviews and syntheses of previously published work while others (*e.g.*, Takeuchi *et al.*, Krug, and Leonard *et al.*) present new data. The paper by Ter Maat *et al.* provides an important comparison of field and laboratory data on reproductive allocation. While this volume may not convey a sense of the beauty and magical atmosphere of the Asilomar Conference Grounds, the papers presented here will provide a sense of the many stimulating directions and developments in the new field of gastropod mating systems research.

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Reproductive behavior of the dioecious tidal snail *Cerithidea rhizophorarum* (Gastropoda: Potamididae)*

Maya Takeuchi, Harumi Ohtaki, and Kiyonori Tomiyama

Department of Earth and Environmental Sciences, Faculty of Science, Kagoshima University, Korimoto, Kagoshima, 890-0065, Japan, cococoro2001@yahoo.co.jp

Abstract: The dioecious snail *Cerithidea rhizophorarum* (Adams, 1855) is distributed along the coasts of the western Pacific up to the Tohoku district, northern Honshu, Japan. It inhabits reed grasslands and mangrove forests with *Kandelia candel* and *Hibiscus hamabo* trees on a mud flat located at the mouth of Atagogawa River in Kiire. Studies of mating and tree climbing behaviors of the species were conducted at this site from April 2000 to May 2003. Mating behavior was observed in July and August 2002. The time of commencement, termination, and duration were recorded for each copulation. The peak of matings during daytime was seen at 1 to 2, and 5 hours before the lowest tide and during nighttime, between 1 hour before and after lowest tide. However, mating rarely occurred on cloudy days. Climbing behavior was observed in an area of 100 square meters where only *K. candel* trees existed. The number of snails on trees was counted, and daily activity of the snails on trees was monitored in summer and winter, hourly throughout the day. The snails were mainly found on mud from spring to summer but frequently climbed up the tree at particular times during summer. Most individuals were on trees and motionless during winter.

Key words: reproduction, dioecious snail, climbing behavior, gastropod

Molluscs have two types of mating systems: dioecy and hermaphroditism (simultaneous hermaphrodite, protandric hermaphrodite, and protogynous hermaphrodite). These divergent patterns of mating systems are found even in the same taxonomic classes. Bisexual reproduction is common in molluscs, and the pattern of fertilization in most classes is internal, but external fertilization is found in some classes. Hermaphroditic species can be divided into ones that can self-fertilize and ones that cannot. Hence molluscs have many divergences in the reproduction pattern and may be the phylum with the most variable reproductive patterns in the animal kingdom. How could such various reproductive strategies evolve?

There are many reports of mating behavior in hermaphrodites such as Pulmonata and Opisthobranchia, but it is rarely reported in dioecious Prosobranchia. A clearer understanding of the mating behavior of this group would be one of the keys to solve the evolution of various reproductive strategies in molluscs.

The dioecious prosobranch snail *Cerithidea rhizophorarum* (Adams, 1855) commonly inhabits tidal flats in eastern Asia. In the tidal flat of the Atagogawa River, Kiire-Cho, Kagoshima, mating by shell mounting was observed (Ohtaki *et al.* 2001). Ohtaki *et al.* (2001) also reported mating behavior, but it was incomplete. In this study, several aspects of mating behavior of *C. rhizophorarum* were examined in the field including duration of copulation both in daytime and nighttime.

MATERIALS AND METHODS

Study site

The tidal flat is at the mouth of Atagogawa River flowing through Kiire-Cho, Kagoshima-city. This river is located by the Nisseki oil camp, and it joins Yahata River in this point. A small mangrove forest consisting of *Kandelia candel* and *Hibiscus hamabo*, at the northern limit of mangrove distribution in the West Pacific, covers this tidal flat. Some species of gastropod, such as *Cerithideopsilla djadjariensis* (K. Martin, 1899), *Cerithideopsilla cingulata* (Gmelin, 1791), *Batillaria multiformis* (Lischke, 1869), *Batillaria cumingi* (Crosse, 1862), *Clypeomorus coralium* (Kiener, 1834), *Reticunassa festiva* (Powy, 1833), *Clithon oualaniensis* (Lesson, 1831), and *Clithon faba* (Sowerby, 1836) inhabit the tidal flat. We established three study sites (A, B, C) 60 m from the shore of Atagogawa River.

Size distribution

We collected 100 *Cerithidea rhizophorarum* with a net (1 mm mesh) at random at the three stations (A, B, C) and

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* From the symposium "Gastropod Mating Systems" presented at the joint meeting of the American Malacological Society and Western Society of Malacologists, held 26-30 June 2005 at Asilomar, Pacific Grove, California.

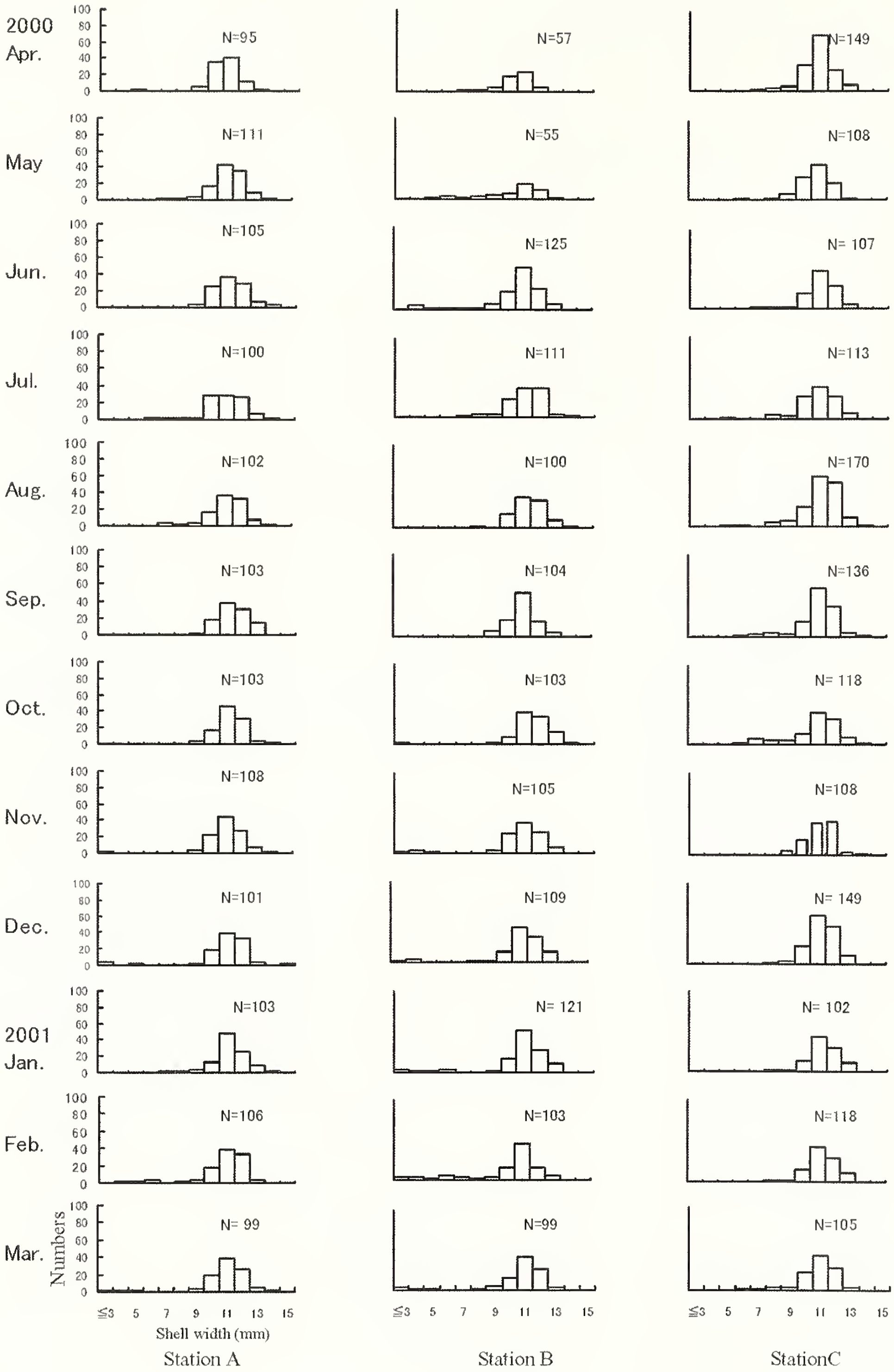


Figure 1. Seasonal change at each station in the width-frequency distribution in *Cerithidea rhizophorarum*.

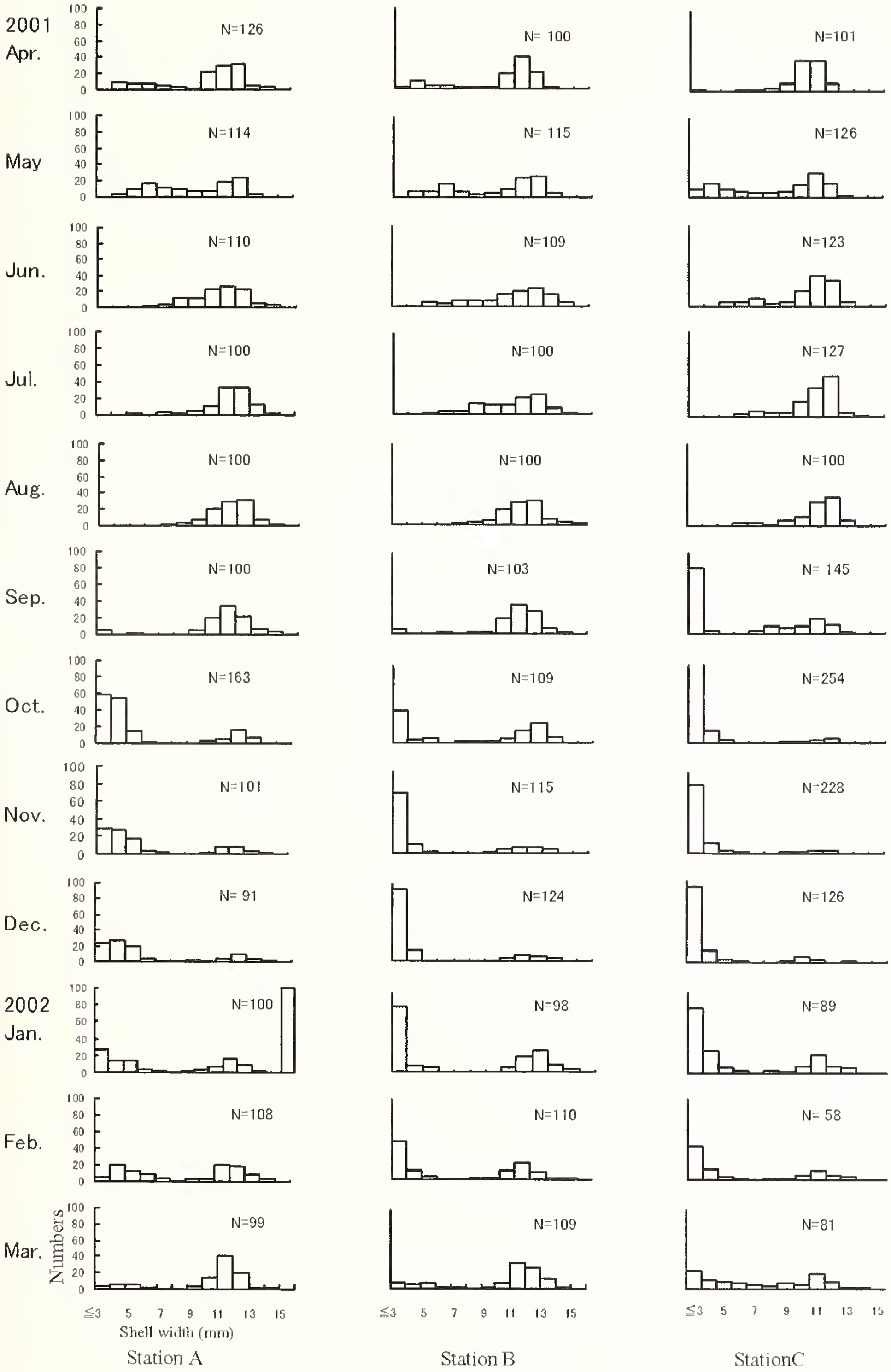
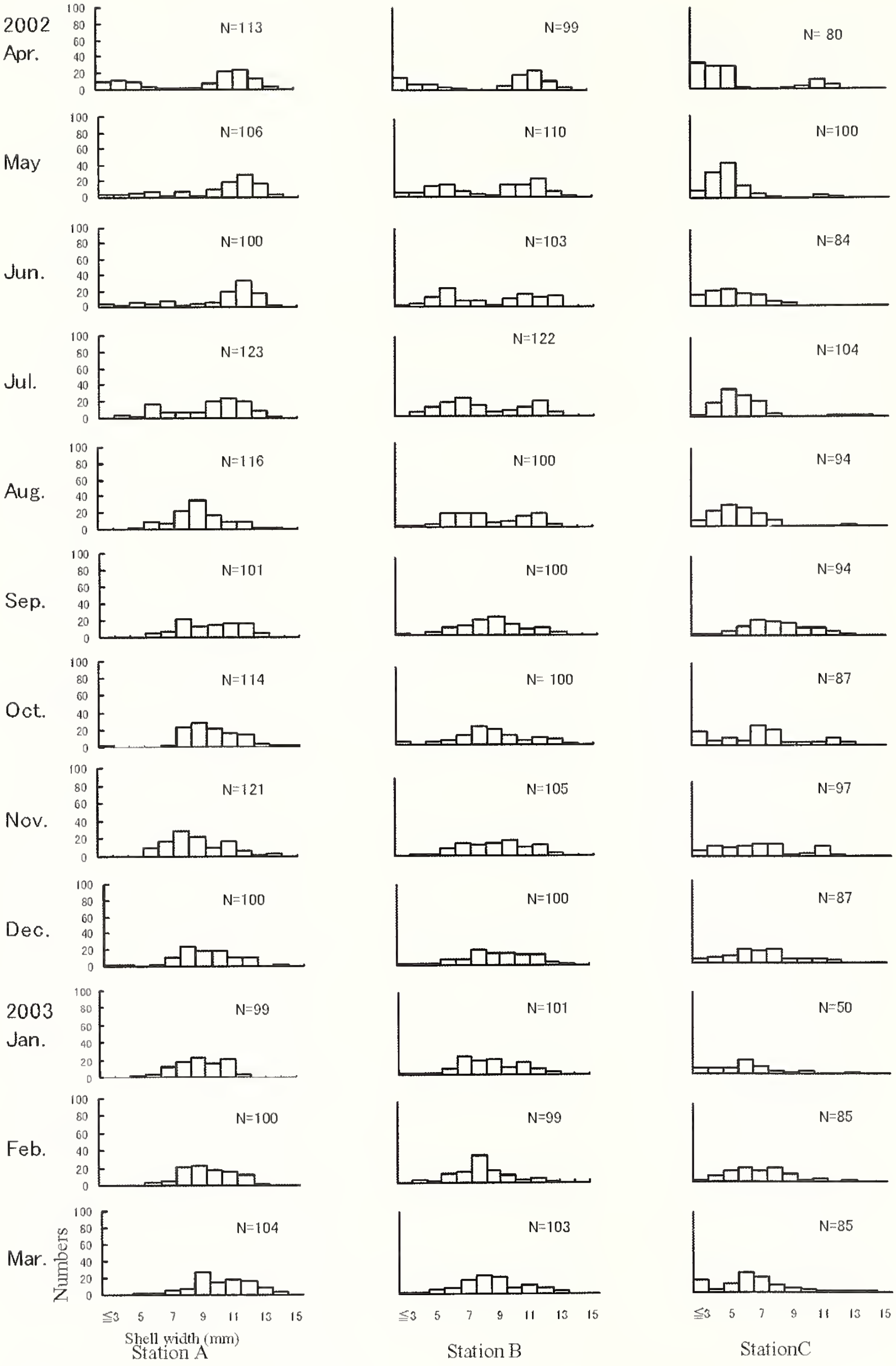


Figure 1. (continued)



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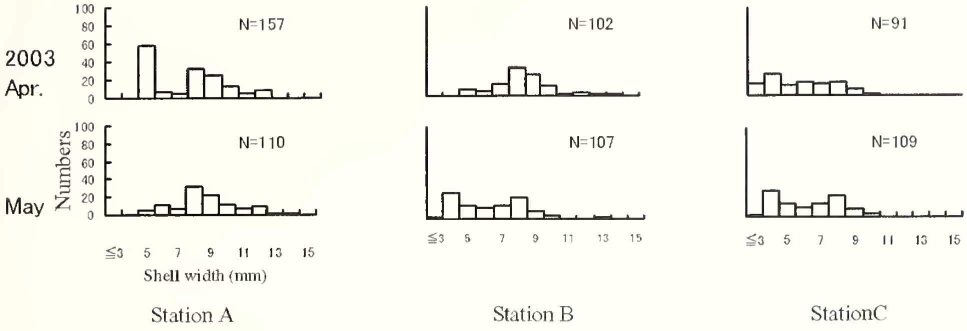

Figure 1. (continued)

measured their shell width *in situ* every month from April 2000 through May 2003.

1. Copulation frequency

Snail mating can be categorized as face-to-face mating or shell-mounting mating (Asami 1998). *Cerithidea rhizophorarum* mates by shell mounting (Ohtaki *et al.* 2001). Ohtaki *et al.* (2001) reported that copulatory behavior was observed from the middle of June to the middle of August and peak of initial time of copulation was before lowest tide. In this study, we counted the number of individuals and copulating couples along the route every 15 minutes on 4 July and 21 July 2001, 12 and 25 June, 12 and 13 July, and 10 August 2002 in daytime, and 12 and 13 July 2002 at night. While sampling the transect every 15 minutes, we placed little flags marked with an identification number near each pair of *C. rhizophorarum* in the act of coupling. When the pair of *C. rhizophorarum* separated, it was assumed that the copulation was terminated. The length between the beginning and the end of the copulation was checked and noted. We used a headlamp for night searches. On the same day, we picked 100 copulation pairs at random and marked upper snails with a permanent marker. We brought copulating pairs back to laboratory and measured them (both weight and shell height). Their shells were broken, and we determined their sex by examining the reproductive gland.

2. Climbing behavior

Cerithidea rhizophorarum is known as an intertidal snail species, but it does climb trees. This behavior was observed in a small mangrove forest of the Atagogawa River (Wakamatu and Tomiyama 2000, Ohtaki *et al.* 2002). In this study, we observed snails on the trees from May 2000 to May 2003, 2 hours after the lowest tide of spring tides every month.

RESULTS

Size distribution

During this period no large change in the number of *Cerithidea rhizophorarum* was noticed (Fig. 1). Newly recruited juveniles (3-6 mm in shell width) of *C. rhizophorarum* were found in higher tidal zones than the other potamidid and batillariid species. The distribution of juveniles of *C. rhizophorarum* was limited to lower tidal zones.

Copulation frequency

The largest number of copulations was observed between one and two hours before the lowest tide in the afternoon and two hours before and after the lowest tide at night (Fig. 2). The mean shell width of upper individuals was 10.96 ± 2.36 mm (mean ± *SD*, $N = 100$, range = 8.6-13.0 mm) and 11.72 ± 29 mm (mean ± *SD*, $N = 100$, range = 8.8-13.9 mm) for lower individuals (Fig. 3). There was no significant correlation between the shell width of upper and lower individuals ($R^2 = 0.082$), but upper individuals were significantly smaller than lower ones (Student's *t*-test, $P < 0.05$). From the reproductive organs of the 100 pairs, 65% were male (upper) - female (lower) pairs, 31% were male - male pairs, 1% female - female pair, and 3% female (upper) - male (lower) pairs were observed. The mean width of individuals with male reproductive organs was 11.49 ± 2.69 mm (mean ± *SD*; $N = 69$, range = 8.8-13.9 mm). There was no relation in shell width between the females and the males (*F*-test; $P \geq 0.05$).

Climbing behavior

There were clear seasonal changes in total number of individuals of *Cerithidea rhizophorarum* on the trunk of the mangrove tree *Kandelia candel* (Fig. 4). In 2000, 1071 snails were observed on trees in May, 1355 in June, 601 in July, 46 snails in August, and in September the number of snails increased to 1041. The following year, from September to December, the number of snails climbing up the trunk of mangrove trees was at its peak, with the number decreasing from February through March. This tendency was seen every year at the same period.

DISCUSSION

Wakamatsu and Tomiyama (2000) reported that recruitment of *Cerithidea rhizophorarum* at this site was so

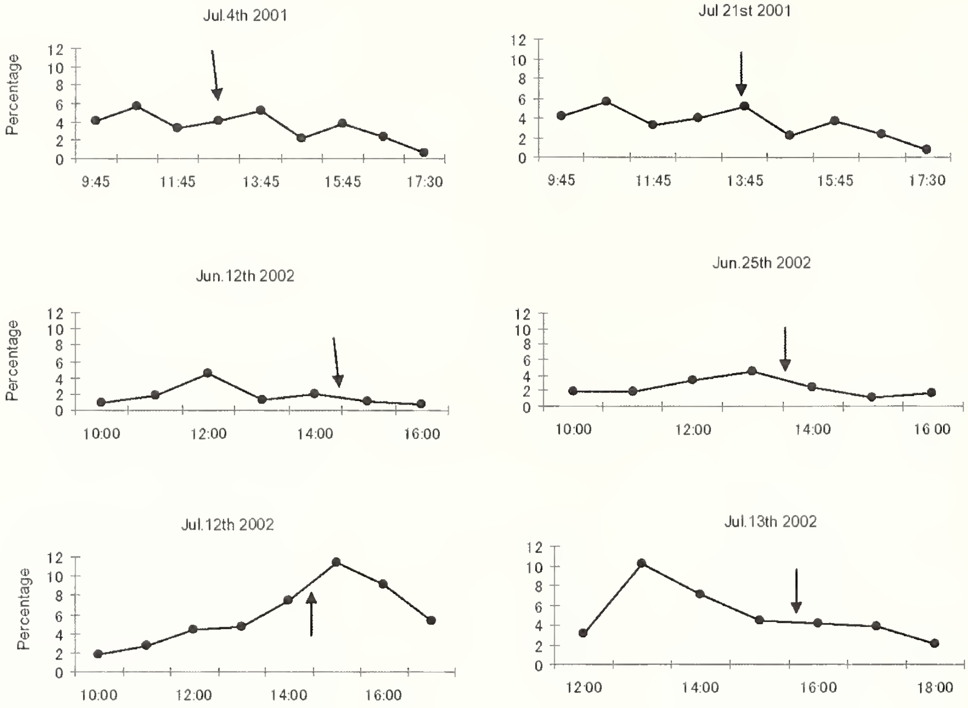

Figure 2. Daily changes in percentage of copulating individuals in the population. Arrows indicate the lowest tides.

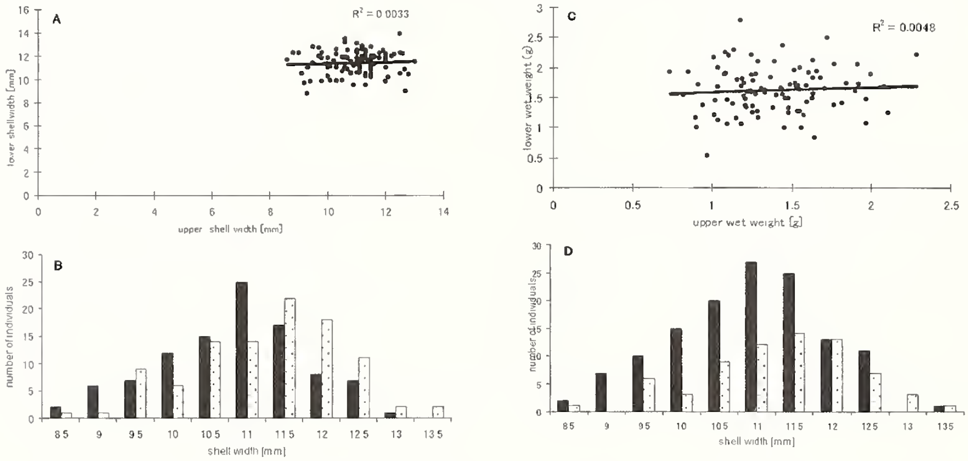

Figure 3. A, Relationship between shell width of upper individuals and lower individuals. $R^2 = 0.0033$, $P > 0.05$, $N = 100$. B, Frequency of shell width of upper individuals and lower individuals. Solid bar, upper individuals; open bar, lower individuals; $N = 100$. C, Relationship between shell width of upper individuals and lower individuals. $R^2 = 0.0048$, $P > 0.05$, $N = 100$. D, Frequency of shell width of male and female. Solid bar, male; open bar, female; $N = 100$.

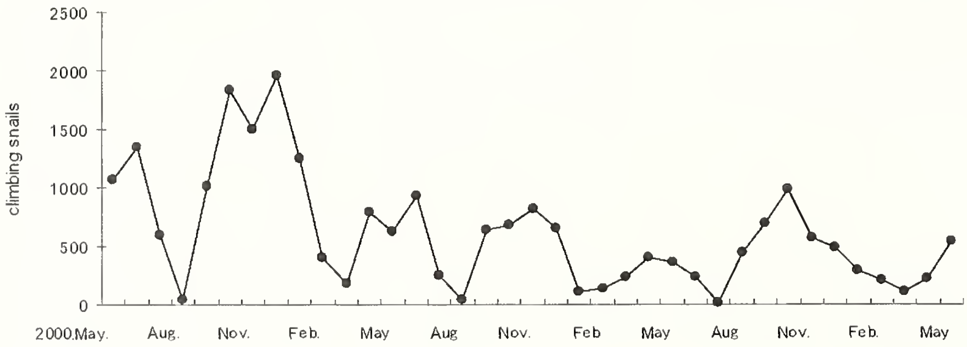

Figure 4. Seasonal changes in total number of individuals of *Cerithidea rhizophorarum* on the trunk of the mangrove tree *Kandelia candel* (May 2000 - May 2003).

small that it could not be detected. But in this study, newly recruited juveniles (3-6 mm) appeared from November 2001 to March 2002, and they grew to 10 mm in length from March to July 2002. However, new recruitment was not observed every year. Wakamatsu and Tomiyama (2000) and Ohtaki *et al.* (2001) suggested that *C. rhizophorarum* had deceased in numbers at this site. We conclude that the decrease in population is due to imposex caused by TBT. From the results of proportions of copulation pairs, it appears males mount females.

More male-to-male (31%) than female-to-female couples were observed (1%), supporting the hypothesis of Wakamatsu and Tomiyama (2000) and Ohtaki *et al.* (2001).

The peak frequency of copulation was generally before the lowest tide in daytime. Copulation was most frequently observed during the 3-6 hours around the time of the lowest tide and mostly during 1-2 hours around the time of the lowest tide at night. As we did not observe copulation when it had begun to rain, rain may block the copulation of *Cerithidea rhizophorarum.*

The population of *Cerithidea rhizophorarum* on trees increased at the highest tide and decreased at the lowest tide. This might be the daily rhythm to avoid the water. Snails were observed on trees from the beginning of spring to June and descended from trees in July to August. These rhythms synchronize with the copulation period. The population on the trees increased in September, and decreased in winter. Climbing trees at the beginning of winter could be in preparation for hibernation.

ACKNOWLEDGEMENTS

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Causes of variation in sex ratio and modes of sex determination in the Mollusca—an overview*

Yoichi Yusa

Faculty of Science, Nara Women's University, Nara 630-8506, Japan, yusa@cc.nara-wu.ac.jp

Abstract: The mechanisms for variation in the primary and apparent sex ratios, from both theoretical and empirical perspectives, are reviewed. A series of experiments on the sex ratios and mode of sex determination in the apple snail *Pomacea canaliculata* (Lamarck, 1822) show that broods have highly variable sex ratios even though the sex ratios of populations are 1:1. I suggest that the mechanism responsible for this pattern is oligogenic sex determination, *i.e.*, sex determination by a small number of genes. Two other molluscan groups, the protandric oysters of the genus *Crassostrea* Sacco, 1897, and mussels of the genus *Mytilus* Linnaeus, 1758 also show variable sex ratios. In both cases, the number of genes responsible for the variation appears to be small.

Key words: sex-determining gene, genetic mechanism, genetic sex determination, molluscs, *Pomacea canaliculata*

Fisher (1930) was the first to show that the sex ratio of a population should be 1:1 if producing a son or a daughter requires an equal cost. Because each offspring inherits half of its genetic material from its mother and half from the father, the members of the sex in short supply will have a higher expectancy of genetic contribution to the next generation than members of the sex in excess. Therefore, the genetic tendencies that produce members of the sex in short supply will be selected by natural selection, until an equal sex ratio is realized in the population.

Since Fisher's theory, studies on sex ratio have expanded along three major lines. The first line is the extension of this theory to include cases for which its premises do not hold, such as local mate competition or local resource competition (Hamilton 1967, Trivers and Willard 1973, Clarke 1978). The second is to treat sexuality in general such as sex allocation in simultaneous hermaphrodites (Charnov 1982). The third treats the conflict between individuals or between genes for reproduction, such as the worker-queen conflict in the Hymenoptera (Trivers and Hare 1976) or conflict between nuclear and cytoplasmic genes (Werren and Beukeboom 1998). Overall, these theoretical studies have fueled many empirical studies, and together they have advanced our understanding of sex ratio or, more generally, of evolutionary patterns (Hardy 2002).

On the other hand, fewer studies have been done on mechanisms of sex-ratio variation. This is probably because of the belief that studying evolutionarily stable sex ratios does not require exact knowledge of the genetic background producing them. However, evolutionarily stable sex ratios are not independent of the underlying mechanisms although they are not fully constrained by the mechanism as evidenced by the presence of large sex-ratio variations under chromosomal sex determination (West and Sheldon 2002).

The sex-determining mechanism is one of the factors affecting sex ratios. However, other genetic or non-genetic factors such as sex-ratio genes, cytoplasmic sex factors, or environmental factors may also affect the sex ratio. On the other hand, the importance of sex determination lies not only in its relevance to sex ratios but also to the problem of sex itself. After all, what sex is and how sex is determined are two of the fundamental questions in biology. Recent studies have succeeded in identifying sex-determining genes (*Sry* in mammals, Sinclair *et al.* 1990; *DMY* in a fish, Matsuda *et al.* 2002). However, most information comes from a limited number of model organisms, and the wide variety of sex-determining mechanisms in many organisms have not been studied. One of the few exceptions is the insightful work on sex-determining mechanisms and its evolution by Bull (1983).

Experimental studies on sex ratio are generally easy to conduct. After all, to study the sex ratio of a population one has only to count the numbers of males and females at an appropriate stage of the life cycle. Considering this, it is surprising how few studies have been done to elucidate the genetic background that produces sex-ratio bias in organisms other than vertebrates and insects. The Mollusca—the most diverse animal taxon in terms of both the number of species and the modes of life, except for the Arthropoda—is no exception. To date, studies on the genetic mechanism producing various sex ratios in the Mollusca are limited to only a few groups, such as the oysters of the genus *Crassostrea* Sacco, 1897 (Haley 1977, Guo *et al.* 1998), the mussels of the genus *Mytilus* Linnaeus, 1758 (Saavedra *et al.* 1997, Kenchington *et al.* 2002), and the apple snail *Pomacea canaliculata* (Lamarck, 1822) (Yusa and Suzuki 2003, Yusa 2004b, 2006, 2007).

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The purpose of this paper is to review studies on the mechanisms that produce sex ratios and the modes of sex determination in molluscs. I do not treat the adaptive significance of sex ratios in detail as there are many good papers such as Hamilton (1967), Charnov (1982), and Hardy (2002).

DEFINITION OF SEX RATIO

I define the sex ratio as the proportion of males / (males + females). I consider the sex ratio in populations consisting mainly of male and female individuals. Thus, in this review I include sex-changing molluscs, as each individual is either male or female at a time. I do not consider simultaneous hermaphrodites, except for treating them as a factor biasing the sex ratio. Charnov (1982) provides the theoretical framework of sex allocation in simultaneous hermaphrodites. Studies on sexual issues in simultaneously hermaphroditic molluscs are reviewed in Leonard (1991), Baur (1998), and other contributions from this symposium.

Sex ratios can be measured at different stages of the life cycle of an organism. Sex ratio at fertilization or when sex is determined is called the primary sex ratio. Sex ratio at birth is called the secondary sex ratio. Sex ratios at later stages can also be considered, such as sex ratio at sexual maturity or that of individuals available for mating (operational sex ratio; Emlen and Oring 1977). In this review, I mainly treat the primary sex ratio. Other sex ratios are referred to as "apparent sex ratios."

Sex ratios can be considered at the population level (population sex ratio) or within each brood (brood or offspring sex ratio). These two are not always the same. For instance, in infinite populations, parents producing any brood sex ratios are equally adaptive although population sex ratio will become 0.5 by Fisherian selection (Williams 1979).

MECHANISMS THAT MAY AFFECT APPARENT SEX RATIOS

Data on sex ratios are often taken from a field population as the proportion of males in adult individuals, without any information on sex differences in mortality, size, or other features. Thus, almost inevitably, many factors potentially affect the apparent sex ratio. To determine the primary sex ratio, well-controlled experiments, where confounding factors are eliminated, are ideal. However, it is often impossible to conduct such experiments, or the field data themselves may be the goal of a study (Takeuchi *et al.* 2007). Even so, many factors may potentially bias the apparent sex ratio (Table 1).

Misidentification of sex

Many molluscs show sexual dimorphism in (i) the shape of the shell or the soft parts (some unionoids, Dillon 2000; the ampullariids *Marisa cornuarietis* [Linnaeus, 1758], Demian and Ibrahim 1972; and *Pomacea canaliculata*, Cazzaniga 1990); (ii) body color (the ampullariid *Marisa cornuarietis*, Demian and Ibrahim 1972); or (iii) body size (ampullariids and viviparids, Dillon 2000; assimineid snails *Assiminea japonica* Martens, 1877 and *Angustassiminea castanea* [Westerlund, 1883], Kurata and Kikuchi 2000). In such cases, the sex may be identified by external morphology without sacrificing the animals. However, the external morphology is often unreliable in identifying sex, and inspection of the gonads or other reproductive organs is preferable whenever possible. Even if gonads are examined, misidentification may occur, especially when only a small amount can be excised for inspection to keep the animals alive (Bauer 1987).

Various sampling biases

Males and females may differ in habitat use, behavior (such as mobility and activity patterns), conspicuousness (in terms of color or brightness), or body size. For instance, in

Table 1. Mechanisms that may affect apparent and primary sex ratios.

1. Mechanisms for apparent sex ratios
    - Misidentification of sex
    - Sampling biases due to differential habitat use, behavior, etc.
    - Differential mortality (embryonic, juvenile, or later)
    - Differential age at maturity

2 Mechanisms for primay sex ratios
  - Sex-ratio genes and cytoplasmic factors
  - Sexual system
    - Parthenogenesis
    - Sex change
    - Simultaneous hermaphroditism
  - Mode of sex determination
    - Environment
    - Sex-determining genes
      - Heterogamety
      - Oligogenes
      - Polygenes

the vivipariid *Viviparus ater* (Cristofori and Jan, 1832), females tend to hibernate earlier than males; thus, the apparent sex ratio of the population in autumn is male-biased (Keller and Ribi 1993). Different habitat use has been suggested as a cause of female-biased sex ratios in another viviparid, *Sinotaia quadrata historica* (Gould, 1859) (Hirai *et al.* 2004). In many snails females grow larger than males (*e.g.*, *Pomacea canaliculata*, Cazzaniga 1990; *Assiminea japonica*, Kurata and Kikuchi 2000) although in some species males are larger (*Angustassiminea castanea*, Kurata and Kikuchi 2000). Sexual dimorphism in size affects the apparent sex ratio if researchers sample larger individuals more often than smaller ones.

Differential mortality

The sex ratio at birth may be different from the primary sex ratio if the hatching rate differs between the sexes. This effect is especially important when the hatching rate is low. However, the effect of differential hatching rate among egg masses can be assessed by studying the correlation between hatching rate and the secondary sex ratio. If there is no significant correlation, then the differential hatching rates are not responsible for the sex-ratio variation among egg masses (Yusa and Suzuki 2003).

Differential mortality in juvenile or adult stages may also skew the sex ratio at later stages (*e.g.*, *Sinotaia quadrata historica*, Hirai *et al.* 2004; *Busycon carica* [Gmelin, 1791], Avise *et al.* 2004). In addition, if age at maturity differs between the sexes, the sex that matures earlier will normally outnumber the sex that matures later due to the higher mortality of the latter before reaching sexual maturity.

MECHANISMS RESPONSIBLE FOR PRIMARY SEX RATIOS

There are three general categories of mechanisms that may affect primary sex ratios (Table 1): sex-ratio genes and cytoplasmic factors, the sexual system (gonochoric, hermaphroditic, or parthenogenetic), and the mode of sex determination.

Sex-ratio genes and cytoplasmic factors

Sex-ratio genes are nuclear genes that are expressed in the parents (the father, the mother, or both) and control the sex ratio of the offspring. For example, X-chromosome drive genes in *Drosophila* skew the proportion of X-carrying sperm during meiosis or fertilization (Hamilton 1967, Stouthamer *et al.* 2002).

Cytoplasmic sex factors or distorters are genetic elements present in the cytoplasm, such as the bacterial genus *Wolbachia* (Stouthamer *et al.* 2002). They often distort the host's sex ratio towards female, because the parasite is usually inherited only through the female lineage and hence female-biased sex ratios are advantageous to them. Cytoplasmic sex factors are not known in Mollusca, and a preliminary trial to detect them was unsuccessful in *Pomacea canaliculata* (Yusa 2006). This does not necessarily mean, however, that all molluscs are free from these factors. A possible candidate, for example, is the paternally inherited (M) mitochondria of *Mytilus* spp. (Saavedra *et al.* 1997, Sutherland *et al.* 1998, Zouros 2000, Kenchington *et al.* 2002, Cao *et al.* 2004) and unionoid bivalves (Dillon 2000).

Sexual system

Parthenogenesis

Molluscs with highly female-biased sex ratios often turn out to reproduce parthenogenetically. For instance, in the freshwater snail *Potamopyrgus antipodarum* (Gray, 1843), the sex ratio varies from 0-50% among populations (Wallace 1992). In most populations the sex ratio is female-biased (Lively 1992). The populations with extremely low sex ratios consist of triploid females that reproduce parthenogenetically, and populations with ratios that are approximately 0.5 consist of diploid sexuals (Lively 1992, Wallace 1992). In some populations, parthenogenetic and sexual individuals coexist; these populations have intermediate sex ratios (Lively 1992, Jokela *et al.* 1997).

Freshwater clams of the genus *Corbicula* Megerle von Mühlfeld, 1811 are predominantly hermaphrodites, reproducing by self-fertilization. However, maternal genes are extruded from the oocyte during the first meiotic division, so that fertilized eggs have only the paternal nuclear genome (androgenesis; Komaru *et al.* 1997, Ishibashi *et al.* 2003). Because the offspring have the same genome as the parent through non-reductional sperm, this represents a special case of parthenogenesis.

Sex change

Sex change is either protandrous (first mature as male and then change sex to female) or protogynous (female to male). The occurrence of sex change has two major effects on sex ratio. First, the brood sex ratio, when followed as a time series, is biased towards the first-maturing sex when they are young, and then skews towards the later-maturing sex as a direct consequence of sex change, as shown in *Crassostrea gigas* (Thunberg, 1793) (Guo *et al.* 1998). Secondly, sex change affects the sex ratio of the population as well. The sex ratio should be distorted towards the first developing sex in sex changers (Charnov and Bull 1989). In fact, several studies have reported male-biased sex ratios in protandrous molluscs (the oysters *Crassostrea* spp., Haley 1977, Guo *et al.* 1998; the pearl oyster *Pinctada mazatlanica* Jameson, 1901, Arnaud-Haond *et al.* 2003; the slipper shell of the genus

Crepidula Lamarck, 1799, Hoagland 1978, Collin 1995, Richard *et al.* 2006).

Sex ratio may vary seasonally or spatially in protandrous molluscs. For instance, in *Crepidula convexa* Say, 1822, the sex ratio is male-biased from fall to spring, when new recruits become sexually mature as males, then female-biased in summer when many of them change sex to female (Hoagland 1978). Hoagland also reported positive correlations between adult density and sex ratio in many local populations of *Crepidula fornicata* (Linnaeus, 1758) and *C. convexa*. This is not due to adaptive sex-ratio adjustment in response to variable density, but rather to variation in the number of recruits among sites: sites with many recruits have higher densities and, after the recruits mature as males, have higher proportions of males.

Simultaneous hermaphroditism

In some organisms, simultaneous hermaphrodites coexist with males (androdioecy), females (gynodioecy), or both (trioecy). In the Mollusca, coexistence of gonochoric individuals and simultaneous hermaphrodites has been reported (unionoid bivalves, Dillon 2000; the freshwater pearl mussel *Margaritifera margaritifera* [Linnaeus, 1758], Bauer 1987; *Mytilus* spp., Saavedra *et al.* 1997). When the proportion of hermaphrodites is small and the population consists mainly of gonochoric individuals, the sex ratios are nearly 0.5 in most species studied so far (for unionoid bivalves, see table 2.2 in Dillon 2000; in *Mytilus*, Saavedra *et al.* 1997). In the freshwater mussel, *Margaritifera margaritifera*, the proportion of males in the population consisting of males, females, and hermaphrodites is nearly 0.5 (Bauer 1987). Because females can change sex to hermaphrodites and *vice versa*, the equal proportion of males and non-males suggests that a simple genetic mechanism such as heterogamety is involved (Bauer 1987).

MODE OF SEX DETERMINATION

Environmental sex determination

In the case of gonochoric organisms, an individual's sex is determined environmentally, genetically, or both. Environmental sex determination can result from factors such as temperature, food availability, daylength, or the presence of adult individuals (Bull 1983). Well-known molluscan examples are slipper shells of the genus *Crepidula*, in which small individuals that settle on larger individuals mature as males, whereas solitary ones mature as females (Hoagland 1978). These small males may change sex, but there are also true males which do not have the ability to change sex (Coe 1936; although Hoagland raises some doubt about this). As an adaptive response, sex ratios in organisms with environmental sex determination are expected to be biased towards the sex developing in the worse conditions (*e.g.*, poor nutrients; Charnov and Bull 1989).

Genetic sex determination

Sex-determining genes can be distinguished by the number of genes into two-factor, oligogenic and polygenic systems. Sex determination by heterogamety, such as XY (male heterogamety) or ZW (female heterogamety), is a well-known system involving two genetic factors. The majority of gonochoric molluscs with known sex-determining systems are XY (gastropods, Nakamura 1986; some bivalves, Allen *et al.* 1986, Guo and Allen 1994). Examples of XO sex determination, with XX females and XO males, are widespread in the Neritidae (Nakamura 1986).

In species of the freshwater snails *Viviparus* Montfort, 1819, both XY and ZW sex determinations are known to occur, although ZW is the majority (Barsiene *et al.* 2000). This has an important implication that one sex-determining system (*e.g.*, XY) can evolve from another (ZW) relatively easily.

Under heterogamety, the expected brood and population sex ratios are both 0.5, with little variation expected under the binomial distribution unless other mechanisms affect the sex ratios. In accordance with this, the sex ratio is 0.5 in most molluscan populations where data are available (*e.g.*, in the unionoid bivalves and in freshwater "prosobranchs", Dillon 2000), although female-biased sex ratios are reported in some "prosobranchs" such as *Pomatiopsis cincinnatiensis* (Lea, 1840) and *Goniobasis* (also referred to as *Elimia* H. & A. Adams, 1854) *semicarinata* (Say, 1829) (Dillon 2000). Unfortunately, due to the lack of sufficient information, the reason for the female-bias is unknown.

Oligogenic sex determination depends upon a small number of genes. For instance, in the platyfish and wood lemming, sex is determined by three genetic factors, X, Y, and W (Bull 1983). Under oligogenic sex determination, offspring sex ratios vary among different pairs of parents. For instance, in the platyfish, mating between an XX female and a YY male produces all sons (XY males), whereas mating between a WX female and an XY male produces only one-quarter sons (Bull 1983).

Polygenic sex determination is a system in which many genes are involved in determining an individual's sex. Each gene has only a minor effect on sex determination, and hence, sex-ratio variation. Models of polygenic sex determination are proposed by Bulmer and Bull (1982) and Bull (1983). There are no molluscan examples of polygenic sex determination.

SEX-RATIO VARIATION AND SEX DETERMINATION IN *POMACEA CANALICULATA*

The apple snail *Pomacea canaliculata* (Ampullariidae) is a South American freshwater snail introduced into many Asian countries, including Taiwan, the Philippines, Thailand, Vietnam, Japan, and China (Naylor 1996, Yusa and Wada 1999). It is a serious rice pest (Naylor 1996) as well as a keystone species controlling the function of wetland ecosystems (Carlsson *et al.* 2004). Because no effective control methods have been developed, sex-ratio variation in *P. canaliculata* has been studied to explore novel genetic control methods (Yusa 2004a, 2004b).

Pomacea canaliculata is gonochoric. Although there is a report that *P. canaliculata* can change sex (Keawjam and Upatham 1990), later authors have failed to confirm this. Sex ratios in populations in rice fields are often female-biased (Banpavichit *et al.* 1994, Tanaka *et al.* 1999), but these may be due to differential survival or growth rates between the sexes.

To study the sex ratio at birth, we removed up to 80 hatchlings from each egg mass, and reared them for 50 days or more, until individuals began developing their reproductive organs (Yusa and Suzuki 2003). We dissected all snails to identify the sexes based on the presence or absence of the testis (for males) or the albumen gland (for females). We used egg masses collected for three months (July, August, and September) in organic rice fields where no chemicals had been sprayed, and those laid by laboratory-reared pairs.

Sex ratios varied greatly among egg masses from nearly all males to nearly all females (Fig. 1). In many egg masses, the sex ratio was significantly different from 0.5. For instance, in July the sex ratio was significantly different ($P <$ 0.05 by binomial test) from 0.5 in 15 out of 27 egg masses. This proportion is much higher than expected by chance (only 1 or 2 out of 27 egg masses are expected to be statistically significant at $P = 0.05$). Likewise, the sex ratio was significantly biased in 13 of 25 egg masses in August, and in 7 of 27 in September.

Irrespective of the large variation among egg masses, the average sex ratio was close to 0.5 in all three months (mean ± SD = 0.47 ± 0.20 in July; 0.53 ± 0.25 in August; and 0.47 ± 0.18 in September). Thus, brood sex ratios of *Pomacea canaliculata* were highly variable, yet population sex ratios were unbiased. We repeated similar experiments using egg masses of laboratory-reared mating pairs originating from two different populations. The results were nearly identical: large variations in brood sex ratios and unbiased population sex ratios were detected in all eight experiments conducted (Yusa and Suzuki 2003).

Survival was high (94-98%) during the rearing period and did not explain the variation in brood sex ratio. Hatching rate was low, but there was no correlation between hatching rate and sex ratio in any experimental series. A possible factor responsible for variation in sex ratio was egg weight. The relationship between the average weight of an egg and the sex ratio was negative in all eight experimental series, and the relationship was significant in three experimental series. Heavier eggs tended to produce female snails whereas lighter ones produced males. Although the meaning of this relationship is unknown, the effect was too small to explain all of the variation.

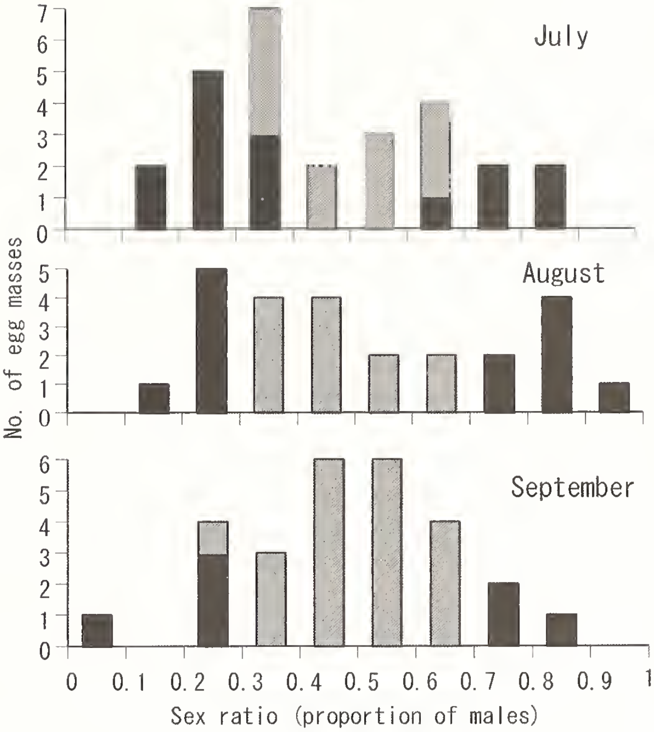

Figure 1. Brood sex ratio (proportion of males) of field-collected egg masses in the apple snail *Pomacea canaliculata* (after Yusa and Suzuki 2003). Black areas indicate egg masses whose sex ratios are significantly different from 0.5 ($P < 0.05$ by binomial test); gray areas indicate those that are not significant.

Environmental effects on sex ratio in *Pomacea canaliculata*

To study the effects of environment on brood sex ratio, I used a split-brood design, where each brood was split into two (or sometimes three) groups of nearly equal numbers of hatchlings, and each group was reared under two different conditions for each environmental factor (Yusa 2004b). I tested factors such as the presence of adult males or females, food availability, temperature, age of the parents, size of the aquarium (as an index of crowding), and indoor or outdoor conditions (with different daylengths).

Among the environmental factors studied, none had a significant effect on brood sex ratio. On the other hand, brood sex ratios were significantly different among different egg masses or parents in all experiments. These results suggest that environmental sex determination does not occur and that the variation in sex ratio is under genetic influence in this species.

Genetics of sex-ratio variation in *Pomacea canaliculata*

To investigate the genetics of sex-ratio variation, I studied parent-offspring regressions and sib correlations of sex ratio (Yusa 2006). For parent-offspring regressions, the sex ratio of each brood was regressed to the sex ratio of its father's siblings or that of its mother's siblings. Correlations in sex ratios between sisters and between brothers were also investigated.

There were significant positive relationships in brood sex ratio in the female lineage: the regression between offspring sex ratio and the sex ratio of the mother's siblings (slope = 0.28) and the correlation between the offspring sex ratios of two sisters (r = 0.41) were both significant ($P <$ 0.05). On the other hand, father-offspring regression (slope = 0.10) and the correlation between two brothers (r = −0.13) were not significant (Yusa 2006).

Thus, the results suggest inheritance of sex-ratio variation through the female lineage. However, the regression or correlation coefficients were too low to postulate cytoplasmic sex factors or maternal genes, which may show coefficients of nearly 1.0 for mother-offspring regression and correlation between sisters (Table 2; Yusa 2006). The results were not congruent with typical sex-ratio genes or sex-determining polygenes with additive effects, which will show coefficients of nearly 0.5 for both male and female lineages (Table 2). A further study suggests that the variation in sex ratio is due to sex determination by a small number of genes (oligogenic sex determination; Yusa, 2007).

Oligogenic sex determination means the coexistence of multiple sex-determining genes in a population. It may occur during the evolutionary transition from one sex-determining system to another, or it may result from a mixture of two or more populations with different sex-determining systems. In *Pomacea canaliculata*, XY sex determination is reported in some Japanese populations (von Brand *et al.* 1990) but not in Brazil (Mercado Laczkó and Lopretto 1998). This discrepancy suggests that different sex-determining systems may coexist in *P. canaliculata*, as they do in the frog *Rana rugosa* Schlegel (Nishioka *et al.* 1994).

GENETICS OF SEX DETERMINATION IN OTHER MOLLUSCS

Sex determination in *Crassostrea*

Oysters of the genus *Crassostrea* change sex. In general, they are protandrous hermaphrodites, but the details of the sexual system differ among species and experimental conditions.

In *Crassostrea virginica* (Gmelin, 1791), the majority of individuals change sex from male to female, but a small proportion of individuals may change from female to male (Haley 1977). Pure males and pure females also exist. Haley proposed an additive 3-loci model to explain the difference in brood sex ratio and patterns of sex between families, with *m* being the allele for maleness and *f* for femaleness. Under this hypothesis the sex of an individual is determined by ratio of *m:f* alleles, such that those with 3-6 *m*'s are true males, those with 2 *m*'s are protandrous hermaphrodites, 1 *m* are females (possibly protogynous hermaphrodites), and no *m* are true females. To my knowledge, this is the only study suggesting oligogenic sex determination in the Mollusca. However, both the experiments and the hypothesis have been criticized by later authors (Guo *et al.* 1998; Yusa and Suzuki 2003).

In *Crassostrea gigas*, individuals are either protandrous hermaphrodites or true males. In a study of parental effects on the sex ratio, Guo *et al.* (1998) found that the sex ratio of

Table 2. Expected regression or correlation coefficients of brood sex ratios under various genetic systems and those actually obtained in *Pomacea canaliculata* (after Yusa 2006). The genes are supposed to have additive effects.

Genetic system	Mother-offspring regression	Father-offspring regression	Correlation between sisters	Correlation between brothers
Cytoplasmic sex factors	1	0	1	0
Sex-ratio genes (biparental)	0.5	0.5	0.25	0.25
Polygenic sex determination	0.5	0.5	0.25	0.25
Data obtained in *P. canaliculata* (mean ± SE)	0.28 ± 0.13	0.10 ± 0.16	0.41 ± 0.18	−0.13 ± 0.19

individuals at one year of age was dependent on the father and independent of the mother. They proposed that the sex is determined by a single locus, with *M* and *F* genes, and individuals of *MF* genotype become males and those with *FF* genotype are protandrous hermaphrodites.

Sex-ratio variation and doubly uniparental inheritance of mitochondria in *Mytilus* spp.

In the mussels *Mytilus edulis* Linnaeus, 1758, *Mytilus trossulus* (Gould, 1850), and *Mytilus galloprovincialis* Lamarck, 1819, brood sex ratios vary from all males to all females (Saavedra *et al.* 1997, Kenchington *et al.* 2002). Irrespective of this variation, the average of brood sex ratios (population sex ratio) is nearly 0.5 (Saavedra *et al.* 1997, Zouros 2000, Kenchington *et al.* 2002). Such highly variable brood sex ratios under equal population sex ratios are similar to the sex-ratio patterns in *Pomacea canaliculata.* The major difference between the mussels and the apple snail is that in the mussels sex ratio appears to be dependent only on the mother and independent of the father (Saavedra *et al.* 1997, Zouros 2000, Kenchington *et al.* 2002; but see below), whereas in the apple snail both parents contribute equally to the sex-ratio variation (Yusa 2007).

In mussels, sex-ratio variation involves a phenomenon called "doubly uniparental inheritance" (DUI) of mitochondria (Saavedra *et al.* 1997, Zouros 2000, Kenchington *et al.* 2002). There are two types of mitochondria, M and F. The M types are transmitted only from the father to the sons through sperm, and the F types from the mother to both sons and daughters through eggs. DUI has been demonstrated in five families of bivalves: Mytilidae, Unionidae, Hyriidae, Margaritiferidae, and Veneridae (Kenchington *et al.* 2002, Cogswell *et al.* 2006, Walker *et al.* 2006).

Saavedra *et al.* (1997) postulate a masculizing factor, W, in M-type mitochondria. This is supported by the observations that (i) some fathers who fail to pass the M genome to their offspring have few sons and many daughters (Saavedra *et al.* 1997), and that (ii) M mitochondria are passed into female embryos as well as male embryos. However, in the female embryos they appear to be outnumbered by F mitochondria whereas in the male embryos M mitochondria preferentially enter into the supposed germ line (Sutherland *et al.* 1998, Cao *et al.* 2004, Cogswell *et al.* 2006). These observations suggest that embryos with many M types in the germ line will develop the testis and become males. Because some fathers fail to pass the M genome to their offspring, at least some part of genetic sex-ratio variation depends on the father. However, this effect is small and the major source of sex-ratio variation depends on the mother (Saavedra *et al.* 1997, Kenchington *et al.* 2002).

The fact that most genetic variation in the sex ratio depends on the mother suggests that some factor from the mother controls the behavior of M mitochondria. Saavedra *et al.* (1997) postulate that there is a feminizing factor Z, and the amount of the factor in the eggs depends on the mother. Kenchington *et al.* (2002) further postulate that the factor is controlled by a pair of alleles (*Z* and *z*) at a single locus. This allows only three genotypes of females: *ZZ*, *Zz*, and *zz*. The *ZZ* mothers produce almost all sons, *Zz* mothers produce both sons and daughters at a fixed ratio, and *zz* mothers produce all daughters (Kenchington *et al.* 2002). However, the simple 2-factor model of a feminizing factor Z does not fully explain the variable brood sex ratios observed in *Mytilus.* Continuous but highly variable sex ratios probably require more factors, at least some of which may be genetic, as suggested by the pedigree experiments (Kenchington *et al.* 2002).

In summary, there appears to be a masculizing factor W in M mitochondria in *Mytilus* spp. Some genetic variation exists in the fathers' ability to pass the M genome to the offspring. Also there may be another sex-determining factor Z in the eggs, which may control the action of W. The genetics of Z factor is unknown, but probably more than two genes or alleles are necessary to explain the large variation in the sex ratio. The genetics of sex-ratio variation in *Mytilus* therefore seems to be oligogenic.

CONCLUSION AND FUTURE DIRECTION

Sex ratio is a basic property of a population and hence has direct relevance to the fitness of the individual controlling the sex ratio of the brood. The sex-determining system may not be fixed, but rather may be fairly labile and subject to evolutionary changes. For example, XY and ZW sex determination coexist in *Viviparus*; there appears to be oligogenic sex determination in *Pomacea canaliculata*, and possibly in *Mytilus* spp.

Studies on variation in sex ratio, such as the ones on *Pomacea canaliculata*, are very easy to conduct—just rear the hatchlings and determine their sexes. Also, studies on sex chromosomes are not difficult, yet very few studies have been done in molluscs, especially outside gastropods. Considering the wide variety of molluscan taxa and their life histories, I suspect that studies may lead to unanticipated findings.

At the same time, novel techniques are useful. For instance, Avise *et al.* (2004) developed molecular sex identification techniques using microsatellite loci in the whelk *Busycon carica.* Also, artificial formation of triploid individuals by cytochalasin B treatment was successful in elucidating detailed mechanisms of XY sex determination, such as Y being dominant to X in the dwarf surfclam *Mulinia lateralis* (Say, 1822) (Guo and Allen 1994), or sex determination by X:autosome ratio in soft-shelled clam *Mya arenaria* (Allen *et al.* 1986). Such techniques will be powerful tools to study the genetics of sex determination and sex-ratio variation in molluscs.

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Poecilogony and larval ecology in the gastropod genus *Alderia**

Patrick J. Krug

Department of Biological Sciences, California State University, Los Angeles, California 90032-8201, U.S.A., pkrug@calstatela.edu

Abstract: The gastropod genus *Alderia* (Allman, 1845) (Opisthobranchia: Sacoglossa) contains a rare case of poecilogony, or variable larval development within a species. This paper reviews the alternative larval morphs and dispersal strategies expressed by *Alderia* spp., and presents new data on larval ecology, environmentally induced changes in development, and rates of metamorphosis for larvae differing in age and life history. Recent morphological and molecular analyses revealed a cryptic poecilogonous species in the previously monotypic genus *Alderia*. The newly described *Alderia willowi* Krug *et al.*, 2007 occurs south of Bodega Harbor, California, and was the subject of all prior studies by Krug and co-workers. Unlike its strictly planktotrophic congener *Alderia modesta* (Lovén, 1844), *A. willowi* produces either small feeding larvae that have a 30-day pre-competent period or large larvae that need not feed to metamorphose. Individuals can vary the developmental mode of their offspring, with a variable proportion switching from lecithotrophy (prevalent in summer) to planktotrophy in winter or spring; a similar shift is induced in some adults upon transfer to the laboratory. In a second dispersal polymorphism, a variable percentage of lecithotrophic larvae undergo spontaneous metamorphosis within 2 days of hatching, while their siblings disperse until induced to settle by carbohydrate cues from the host algae *Vaucheria* spp. The percentage of spontaneous metamorphosis is uncorrelated with fecundity and is generally between 15-30%, a possible product of stabilizing selection on this bet-hedging dispersal strategy. Despite their different ages, competent larval morphs produced by alternative developmental pathways are similar in size, swimming behavior, and responses to dissolved settlement cues. However, competent planktotrophic larvae and older lecithotrophic larvae initiated metamorphosis faster after settlement than newly hatched lecithotrophic larvae, suggesting a link between planktonic period and habitat choice. Although rare, poecilogonous species like *A. willowi* offer special insights into the evolutionary causes and ecological consequences of alternative life histories.

Key words: bet-hedging, cryptic species, dispersal polymorphism, larval settlement, sacoglossan

A major difference between marine and terrestrial life histories is the dichotomy between feeding and non-feeding modes of larval development among marine invertebrates (Strathmann 1990, 1993). Most species produce either a large number of small larvae that must feed in order to attain competence (planktotrophy) or fewer, larger larvae that can metamorphose without feeding (lecithotrophy) (Wray and Raff 1991, Levin and Bridges 1995). Ecologically similar and related species often differ in developmental mode for reasons that remain unclear, and there is no theory specifying which selective regimes should favor lecithotrophy over planktotrophy (Todd *et al.* 1998, McEdward 2000). Phylogenetic studies have demonstrated that transitions between developmental modes have occurred numerous times in diverse groups such as molluscs (Duda and Palumbi 1999, Meyer 2003, Collin 2004), echinoderms (Hart *et al.* 1997, Hart 2000, Jeffrey *et al.* 2003), and urochordates (Hadfield *et al.* 1995). Planktotrophy is presumed to be ancestral to lecithotrophy because complex feeding structures, once lost, are rarely regained via adaptive evolution (Strathmann 1978, 1985, 1993, Wray 1995). Both the fossil record and phylogenetic hypotheses generally support this one-way trend towards non-planktotrophic development (Hansen 1982), although species with nurse eggs may be an exception (Collin 2004). The forces that drive transitions to non-feeding larvae are poorly understood, however, and the dearth of intermediate stages or organisms expressing multiple forms of development within a species have impaired attempts to understand this aspect of life-history evolution.

Although causal explanations remain elusive, the ecological and evolutionary consequences of different developmental strategies are profound (Perron 1986, Pechenik 1999). The paleontological record for molluscs indicates that species with planktotrophic larvae have greater geological longevity and broader geographical ranges than those exhibiting lecithotrophy, which speciate at a higher rate (Hansen 1978, 1980, 1982, Jablonksi 1986). Species with lecithotrophic or direct development are more prone to extinction, but often show greater local adaptation (Hansen 1978, Sanford *et al.* 2003). The prolonged pelagic period of most feeding larvae makes them effective vectors for gene flow, and planktotrophic taxa generally exhibit panmictic populations (Palumbi 1995, Caley *et al.* 1996). The continuous influx of alleles from distant populations can prevent planktotrophic species from adapting to local conditions (Vermeij 1982).

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Intraspecific phylogeographic breaks occur in planktotrophic species but are usually small in magnitude compared to the highly structured populations often observed within species that have non-feeding larvae with reduced dispersal potential (Kyle and Boulding 2000, Wares *et al.* 2001, Collin 2003).

An improved understanding of how selection drives developmental evolution may come from studies of rare species that display intraspecific polymorphism in larval development. Termed "poecilogony", this phenomenon appears perplexingly restricted to spionid polychaetes and opisthobranch molluscs (Giard 1905, Hoagland and Robertson 1988, Bouchet 1989, Chia *et al.* 1996). Molecular studies discredited other purported examples of variable development as cryptic species. True cases of poecilogony can inform as to the ecological significance of alternative dispersal strategies (Levin 1984a, Levin and Huggett 1990, Levin and Bridges 1994), and may indicate when natural selection favors one larval mode over another. Such species may also provide insight into the mechanisms underlying developmental transitions, such as allocation of maternal resources and gene expression (Villinski *et al.* 2002, Marsh and Fielman 2005).

Some opisthobranchs have been classified as poecilogonous because offspring can metamorphose either before or after hatching from benthic egg masses. These may be more constructively termed "dispersal polymorphisms"; they do not constitute variable development as there is no difference in egg size or larval trophic mode. Such dimorphisms can result if the egg mass matrix retains or releases larvae according to season (*e.g.*, *Elysia timida* Risso, 1818; Marin and Ros 1989), adult nutritional state (*Tenellia adspersa* Nordmann, 1845; Chester 1996), or experimental conditions (*Berghia verrucicornis* Costa, 1864; Carroll and Kempf 1990). In the cephalaspidean *Haminaea callidegenita* Gibson and Chia, 1989, some larvae attain competence prior to hatching and undergo intracapsular metamorphosis triggered by an inducer in the egg jelly, while siblings become competent after hatching and settle in response to benthic biofilms (Gibson and Chia 1989). Dispersal polymorphisms can also result from differences in settlement cue requirements among pelagic larvae (Mackay and Doyle 1978, Raimondi and Keough 1990, Toonen and Pawlik 1994), but this has received little attention as a potentially important adaptation in marine life histories.

Truly variable development is expressed in species where developmental mode differs between offspring due to variation in egg size or pre-hatching consumption of nurse eggs or extra-zygotic yolk. In all recognized cases, this involves alternative developmental pathways that produce either feeding larvae that ingest planktonic food after release or larvae that can metamorphose without feeding after release from parental brood structures or benthic egg masses. One definition of "lecithotrophy" applies to larvae that are incapable of ingesting particulate food (Chia 1974); other definitions include larvae that do not need to feed to complete metamorphosis, whether or not they can (Thompson 1967). The larger larval morph of most poecilogonous species can feed, either by ingesting nurse eggs prior to hatching or facultatively capturing phytoplankton if food is available (Gibson and Gibson 2004, Botello and Krug 2006, Pernet and McArthur 2006). In this review, I use Thompson's (1967) definition of "lecithotrophic."

It is noteworthy that no example of variable development involves a free-spawning organism; among polychaetes, eggs are retained in structures associated with adult tubes, and in sacoglossan opisthobranchs, within benthic egg masses. Although there are reported examples of poecilogony among sacoglossans that include pelagic lecithotrophy and direct benthic development (usually through encapsulated metamorphosis), these likely constitute examples of dispersal polymorphisms as described above, or cryptic species (*e.g.*, "*Elysia cauze*," later recognized as three distinct species; Clark and Goetzfried 1978, Clark 1984, 1994).

Research into poecilogony has been historically confounded by the prevalence of cryptic species in the taxa that contain well-documented examples of variable development, namely polychaetes and opisthobranchs (Grassle and Grassle 1976, Clark 1984, Hoagland and Robertson 1988, Morrow *et al.* 1992, Clark 1994, Chia *et al.* 1996, Schulze *et al.* 2000, Kruse *et al.* 2003). Confirmation of variable development requires molecular data or breeding experiments to demonstrate that individuals differing in larval trophic mode are conspecifics. Among polychaetes, such data exist for *Boccardia proboscidea* (Gibson *et al.* 1999, Gibson and Gibson 2004), populations of *Streblospio benedicti* from the east coast of North America (Levin 1984b, Levin *et al.* 1991, Schulze *et al.* 2000), and *Pygospio elegans* (Morgan *et al.* 1999). Among opisthobranchs in the order Sacoglossa, three poecilogonous species have been confirmed by molecular data or laboratory crosses. In breeding studies, planktotrophic and direct-developing specimens of *Elysia chlorotica* Gould, 1870 from different populations produce viable hybrid offspring (West *et al.* 1984). The Caribbean species *Costasiella ocellifera* Simroth, 1895 was reported as a cryptic species complex (Miles and Clark 2002), expressing planktotrophy or benthic development in different populations, but recent data indicate these populations comprise a single species and that individuals differing in development co-occur and share mitochondrial haplotypes (Ellingson and Krug, unpubl. obs.).

The third example of poecilogony among sacoglossans was reported from southern California among sea slugs in the genus *Alderia* (Allman, 1845), a member of the cerata-bearing family Limapontiidae (Krug 1998). *Alderia* spp. are specialized herbivores that feed solely upon the algae *Vaucheria* spp. (Ochrophyta: Vaucheriales), found on mudflats in temperate estuaries throughout the northern hemisphere (Trowbridge 2002). The type species *A. modesta* (Lovén, 1844) was described from northern Europe and later reported from both coasts of North America (Engel *et al.* 1940, Hand and Steinberg 1955, Hartog 1959, Bleakney and Bailey 1967, Clark 1975, Vader 1981, Bleakney 1988, Trowbridge 1993, 2002, Martynov *et al.* 2006). Planktotrophy was the sole developmental mode reported from the western Atlantic (Clark 1975), eastern Atlantic (Engel *et al.* 1940, Seelemann 1967), western Pacific (Chernyshev and Chaban 2005), and eastern Pacific as far south as Monterey, California (Hand and Steinberg 1955, Trowbridge 1993, Gibson and Chia 1994). Populations south of Monterey were recently described as a new species *A. willowi* Krug *et al.*, 2007, based on morphology, molecular data, and the expression of both lecithotrophy and planktotrophy (Ellingson and Krug 2006, Krug *et al.* 2007).

This paper will focus on *Alderia* spp., reviewing prior studies that used *A. willowi* as a model to study swimming and settlement behavior of alternative larval morphs and recent work on cryptic speciation in the genus. New data will be presented to address the following research questions: (1) How do reproductive and larval characteristics for the poecilogonous *A. willowi* compare with those of its planktotrophic congener *A. modesta*? (2) Does environment affect the type of larvae produced by *A. willowi*? (3) Does the percentage of spontaneous metamorphosis co-vary with fecundity or does it remain similar across populations of *A. willowi?* (4) Do newly competent planktotrophic larvae show an accelerated rate of metamorphosis similar to that of older lecithotrophic larvae?

MATERIALS AND METHODS

Study sites and taxa

Specimens of *Alderia* spp. and algae were collected at low tide on mudflats from study sites along the west coast of the U.S.A. (Fig. 1). The algal host in southern California is recognized as *Vaucheria longicaulis* (Abbott and Hollenberg 1976); however, this nominal taxon likely comprises a cryptic species complex, as there has been no detailed taxonomic assessment of *Vaucheria* spp. from the northeastern Pacific. I refer to algae from southern California as *V. longicaulis* but, when referring to the whole coast, use *Vaucheria* spp. to reflect taxonomic uncertainty in this genus.

Clutch and larval characteristics of *Alderia* spp.

For *Alderia modesta,* adults were collected in Bodega Harbor, California, in September and October 2004-2005. Egg masses collected within 8 hr of deposition were isolated in dishes of 0.22-µm filtered seawater (FSW) until hatching commenced. The diameters of eggs and capsules were measured from high-resolution digital photographs of egg strings removed from the egg mass, calibrating pixel number per µm with photographs of a hemocytometer grid at the same magnification. Data for planktotrophic and lecithotrophic development in *Alderia willowi* are from Krug (1998).

Changes in developmental mode: field surveys and laboratory experiments

Surveys of developmental mode were carried out in San Diego (1996-1999, 2003-2004), the Upper Newport Bay Ecological Reserve (2003-2004), and a man-made wetland adjacent to the Los Angeles Harbor (2003-2004). Specimens were isolated in petri dishes of seawater for clutch deposition overnight, and clutches were scored for developmental mode 1-2 days later based on egg size (Krug 1998). The proportion of the population producing lecithotrophic clutches was compared for March *vs.* August of 1996-1999 for San Diego, the only site for which such data were available each year, using a Mann-Whitney *U*-test (as implemented by StatView statistical software package).

Adults collected from Los Angeles were typed for developmental mode of their first clutch (February 2004). Those producing lecithotrophic eggs ($n = 20$) were placed on a patch of *Vaucheria longicaulis* from which all slugs had been removed; algae and slugs were maintained in an incubator at 22 °C on a 14:10 light:dark cycle. Egg masses deposited overnight were collected and typed for developmental mode every 1-2 days for 3 weeks. The proportion of lecithotrophic clutches (y) was regressed against time (x) using a Model 1 regression in StatView.

Spontaneous metamorphosis in clutches of field-collected adults

Adult slugs were collected in San Diego (August 1999), San Francisco (September 2004), and from three sites within Tomales Bay: south Tomales, September 2003; Cow Landing, September 2004, and Walker Creek, September 2004. Slugs were transported to the lab, isolated, and their first clutch harvested. Egg masses were kept in FSW until hatching (5-6 days) and scored for (1) egg number and (2) the percentage of spontaneous metamorphosis occurring in the first 2 days post-hatching (Krug 2001). The null hypothesis of no variation in the percentage of spontaneous metamorphosis between sites was evaluated using a non-parametric Kruskal-Wallis test. A Spearman Rank Correlation test was used to compare fecundity and percentage of spontaneous metamorphosis for the five sites.

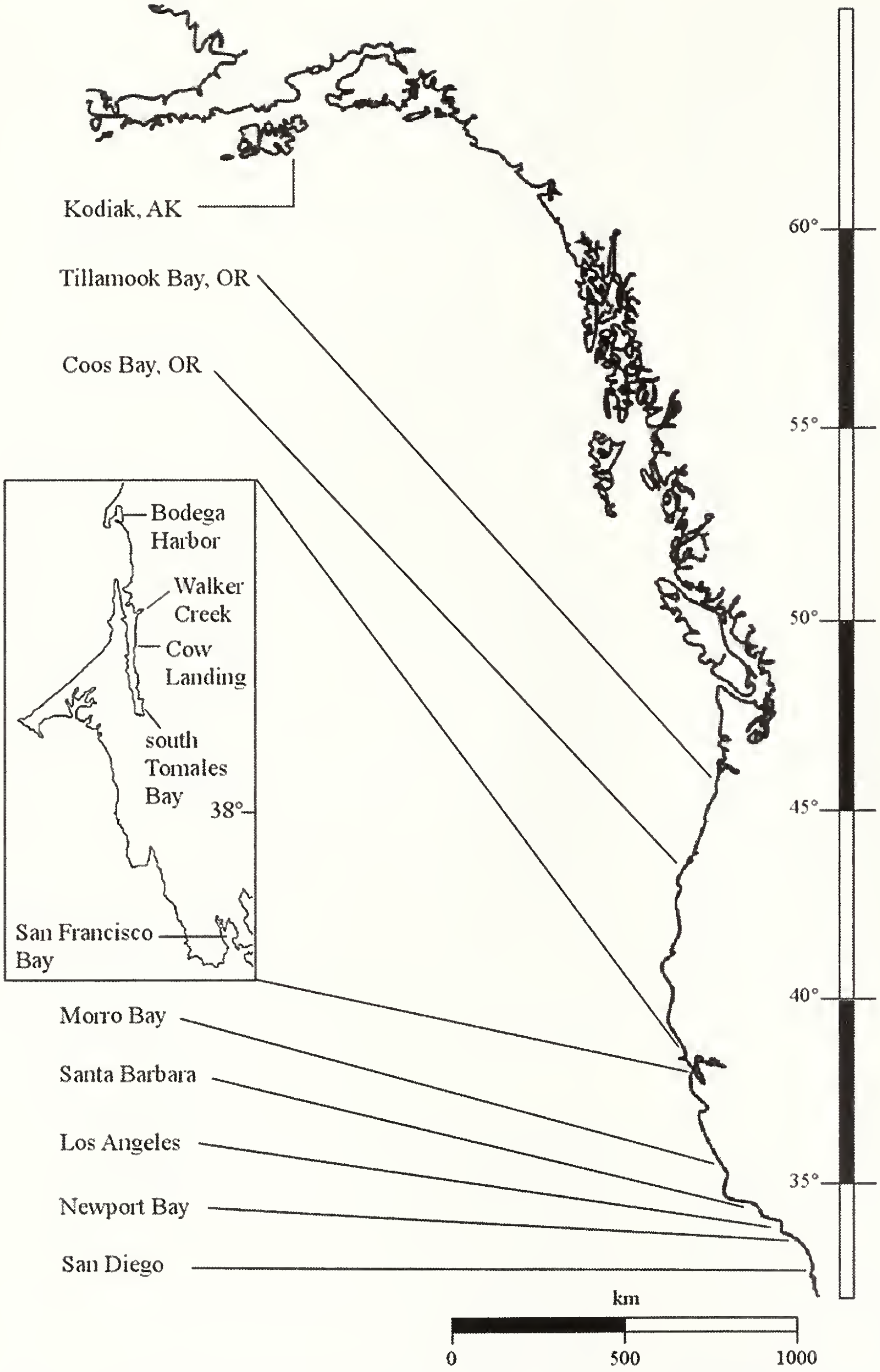

Figure 1. Map of collection sites for *Alderia* spp. from the northeastern Pacific. Inset shows close-up of the area between San Francisco Bay and Bodega Harbor, where both species seasonally co-occur and have their southern (*A. modesta*) or northern (*A. willowi*) range limits.

Rate of metamorphosis in larvae of different ages and developmental modes

Planktotrophic larvae of *Alderia willowi* were cultured as in Krug and Zimmer (2000, 2004) and used in assays after 32 days. Larvae (n = 15 per dish, 6 replicates per age class) were exposed to live *Vaucheria longicaulis* and scored for settlement and metamorphosis starting 2 hr after algae were added and at 12 hr intervals thereafter. Settlement is a reversible attachment to the bottom, whereas metamorphosis is an irreversible transformation into a juvenile slug. Cumulative percentages of metamorphosis were not normally distributed after transformation and were, thus, compared after 24 and 48 hr within each age class using a Wilcoxon Signed Rank test; no difference would indicate that most metamorphosis occurred in the first 24 hr whereas a difference would indicate a delayed metamorphic response. These assays were run concurrently with the experiments in Botello and Krug (2006), a study from which data for lecithotrophic larvae of *A. willowi* aged 1, 2 and 4 days post-hatching were taken; the data for planktotrophic larvae were not previously reported.

RESULTS AND DISCUSSION

Poecilogony and cryptic species in the genus *Alderia*

The first report of lecithotrophy in "*Alderia modesta*" was also a southern range extension of ~500 km for this previously monotypic genus (Krug 1998). The global phylogeography of *Alderia* was recently studied by sampling estuaries throughout the northeastern Pacific (Fig. 1), one site in the western Pacific, and one in the eastern Atlantic. Slugs were typed for developmental mode and morphology of the dorsum and radula, and portions of two mitochondrial genes (cytochrome oxidase I (COI) and 16S ribosomal subunit) were sequenced and analyzed (Ellingson and Krug 2006). Most specimens south of Bodega Harbor, California, U.S.A. comprise a poecilogonous cryptic species, *Alderia willowi* (Krug *et al.* 2007). It is distinguished from *A. modesta* by a humped dorsum covered with fused patches of dark pigment (Figs. 2A-B), the seasonal production of lecithotrophic larvae, and the morphology of its radula and egg masses. The congener *A. modesta* occurs seasonally in San Francisco Bay and from Tomales Bay north throughout the Pacific and north Atlantic. *Alderia modesta* has a smooth, speckled dorsum (Figs. 2C-D) and expresses only planktotrophic development.

Molecular data discriminated clearly between the sister species, which form reciprocally monophyletic clades 18-24% divergent in COI and 2.9% divergent in 16S (Tamura-Nei distances) (Ellingson and Krug 2006). All prior publications by Krug and co-workers on "*Alderia modesta*" in fact concerned its cryptic sister species *A. willowi*. Though nominally conspecific, *A. modesta* from the north Atlantic are substantially divergent (10-12% in COI) from Pacific *A. modesta*. Populations in the two oceans have likely been allopatric since the early Pleistocene, supporting the hypothesis of Bleakney (1988) that trans-Arctic gene flow in *A. modesta* ceased when glaciation covered the Arctic sea. However, given the lack of morphological and developmental differences between ocean basins, *A. modesta* may still be one biological species; breeding studies and further sampling of Atlantic sites are needed to test this hypothesis. Speciation in

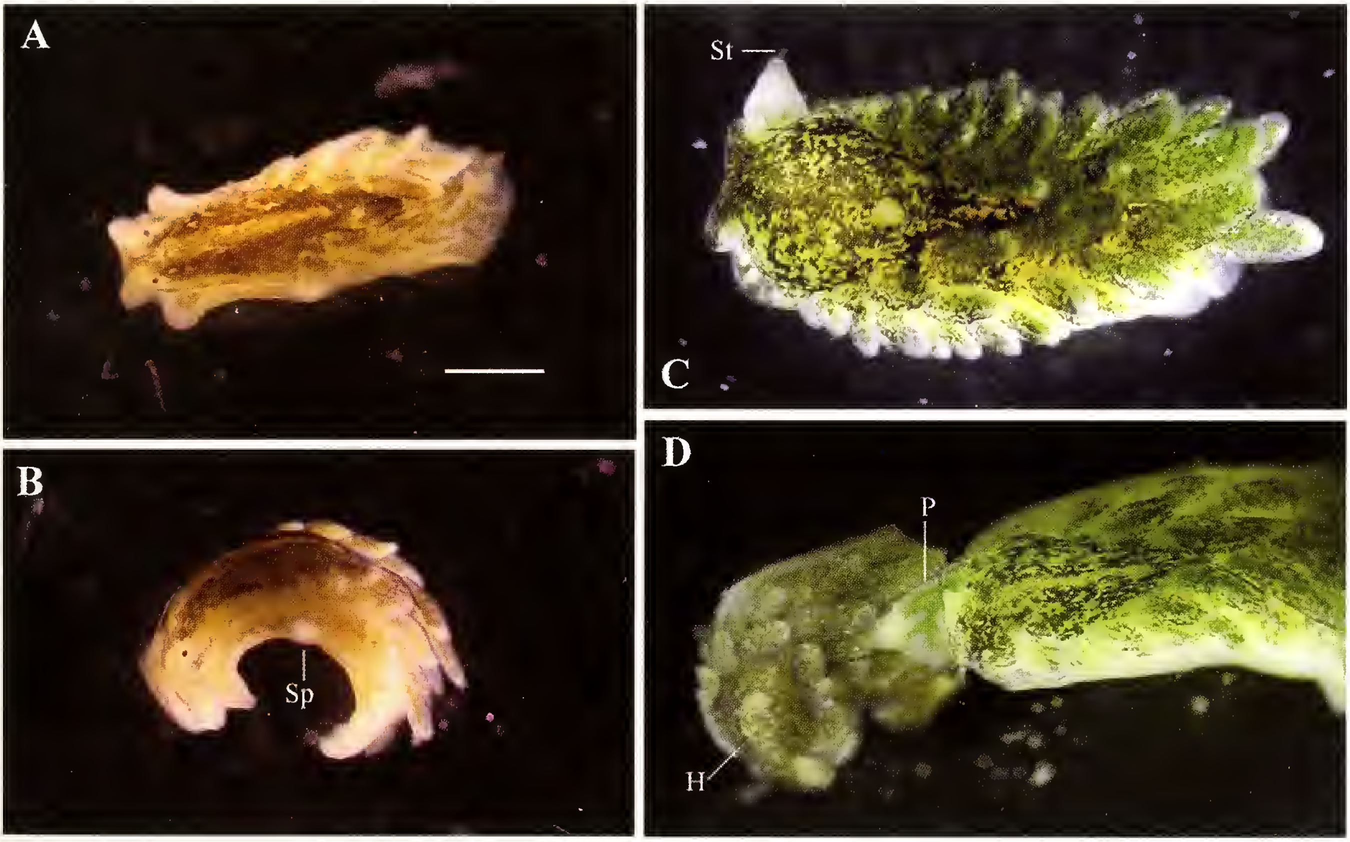

Figure 2. Photographs of individuals of *Alderia* spp. from the northeastern Pacific. A, Dorsal view of *Alderia willowi*, recently described from California. A distinctive morphological feature of this species is that the dorsum is raised into a hump, with a yellow stripe extending down the midline; the dark pigmentation is also fused into continuous patches. B, Side view of the same specimen, which had just been hypodermically inseminated; the white bulge on the foot (Sp) is a subcutaneous sperm deposit. C, Dorsal view of *Alderia modesta*, found on eastern and western coasts of the Atlantic and Pacific. Its dorsum is smooth, often shield-shaped, and covered in speckles of dark pigment. This individual had recently fed on *Vaucheria longicaulis* and the green cytoplasm is visible within digestive diverticula ramifying throughout the body, including the cerata. The penis, extending from the right side of the head, is tipped with a stylet (St) for piercing the body wall of mates during hypodermic insemination. D, Interspecific mating attempt, with a specimen of *A. modesta* probing a smaller specimen of *A. willowi* with its penis (P). Green digestive diverticula can be seen enervating the penis of *A. modesta*. The humped dorsum (H) of *A. willowi* is evident. All photos to same scale; scale bar = 800 μm.

Table 1. Clutch characteristics of *Alderia willowi*, a poecilogonous species occurring south of Bodega Harbor, California, and its plankotrophic congener *Alderia modesta*, which occurs from San Francisco Bay northwards. For *A. willowi*, adults were collected from the Northern Wildlife Preserve in Mission Bay, San Diego, from January to April in 1996 and 1997; plankotrophic and lecithotrophic clutches were reared at 25°C (Krug 1998). Data for *A. modesta* were obtained in September 2005 for specimens from Bodega Harbor. Data are means ± *SD*.

	Alderia willowi		*Alderia modesta*
	Lecithotrophic	Planktotrophic	Plankotrophic
Encapsulated period (days)	5.4 ± 0.6 ($n = 30$)	3.0 ± 0.5 ($n = 30$)	4.7 ± 1.2 ($n = 18$)
Eggs per clutch	32 ± 12 ($n = 30$)	311 ± 134 ($n = 30$)	463 ± 117 ($n = 15$)
Egg diameter (μm)	105 ± 5 ($n = 328$)	68 ± 4 ($n = 517$)	78 ± 4 ($n = 75$)
Egg capsule diameter (μm)	247 ± 31 ($n = 328$)	121 ± 12 ($n = 517$)	127 ± 10 ($n = 75$)
Maximum larval shell dimension at hatching (μm)	186 ± 9 ($n = 282$)	116 ± 8 ($n = 160$)	124 ± 8 ($n = 45$)

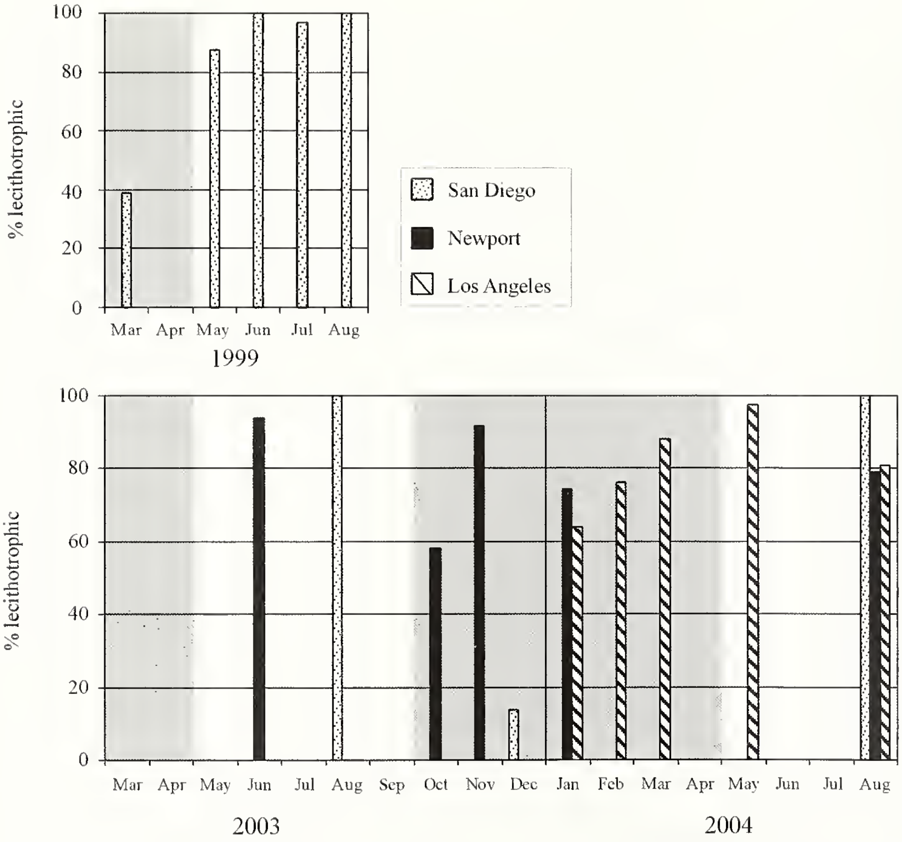

Figure 3. Seasonal variation in the proportion of adult *Alderia willowi* producing lecithotrophic clutches in three southern Californian estuaries. Expression of lecithotrophy generally only drops below 80% from October to April (shaded).

the Pacific pre-dated colonization of the Atlantic, indicating a Pacific origin for the genus *Alderia* (Ellingson and Krug 2006).

Reproductive and larval characteristics of *Alderia* spp.

Developmental data for planktotrophic larvae of *Alderia modesta*, and for both clutch types in *Alderia willowi*, are given in Table 1. This table is adapted from Krug (1998) to reflect the new species status of *A. willowi* and to include comparative data for the true *A. modesta*. The larger planktotrophic clutches of *A. modesta* took longer to develop and hatch than the smaller planktotrophic clutches of *A. willowi*. Mean adult size was greater for individuals of *A. modesta* (range: 5.5 – 23.1 mg, $n = 6$ populations) than for *A. willowi* (range: 0.9 – 5.8 mg, $n = 6$ populations). In both species about 80% of the variance in fecundity is due to adult size (Ellingson and Krug 2006).

Environmentally cued change in development in *Alderia willowi*

Populations of *Alderia willowi* were surveyed in southern California starting in 1996, and showed a consistent seasonal trend. In 1999 (San Diego) and 2003-2004 (3 field sites), clutches were 80-100% lecithotrophic from May-September, but typically less than 80% lecithotrophic from October-April (Fig. 3). The San Diego population had the most extreme shifts; from 1996-1999, the population was 100% lecithotrophic in August but only 55 ± 25% *SD* lecithotrophic in March of those years (Mann-Whitney *U*-test, $Z = -2.3$, $P < 0.05$). The switch to planktotrophy was roughly coincident with seasonal drops in sea-surface temperature and salinity. Populations from Newport Bay and Los Angeles Harbor exhibited a similar trend, but a higher proportion of adults remained lecithotrophic from fall to spring (Fig. 3).

Some slugs expressing lecithotrophy in the field switched to planktotrophy when held in the laboratory for 1-3 weeks (Fig. 4). Adults were maintained on patches of *Vaucheria longicaulis* in the laboratory and laid typical numbers of clutches; the change in their egg size was thus not due to starvation, which can also trigger a switch to planktotrophy (Krug 1998). The proportion of planktotrophic clutches increased over time (Fig. 4, and results of a regression of the effect of time, x, on % planktotrophy, y: $y = -2.80x + 93.69$; $F_{1,9} = 28.18$, $P = 0.0005$, $r^2 = 0.73$). The change in larval type was interpreted as an adult response to the lab environment; reverse transitions to lecithotrophy have not been successfully induced, suggesting the laboratory mimics winter conditions. Siblings reared from the egg under laboratory conditions exhibited either developmental mode and rarely changed between modes (N. Smolensky and P. Krug, unpubl. obs.). Slugs transitioning between development modes in the laboratory sometimes produced clutches of intermediate-sized eggs, first reported in Krug (1998); such clutches are produced by field-collected slugs during transitional months, when the populations are switching either from or towards lecithotrophy (unpubl. data). These data suggest seasonal changes in development reflect changes within individuals, not just between generations within a population.

Together, the evidence suggests that individuals respond to fluctuating conditions by varying their egg size and, hence, mode of development of their larvae. Seasonal shifts in development have not been reported among other poecilogonous species, in which larval type varies by location (West *et al.* 1984, Levin and Huggett 1990, Levin and Creed 1991, Morgan *et al.* 1999, Miles and Clark 2002). There is no other example of a poecilogonous species in which individual adults facultatively change the larval type of their offspring, making *Alderia willowi* a unique model system for investigating the ecological forces and biochemical mechanisms underlying alternative developmental modes.

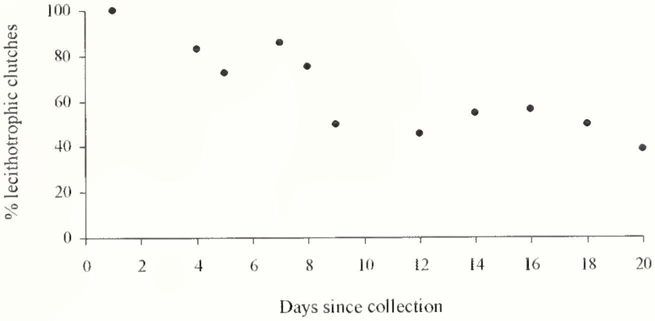

Figure 4. Change in developmental mode expressed by adult *Alderia willowi* following collection from the field. Adults initially produced lecithotrophic larvae when first collected and were maintained on patches of *Vaucheria longicaulis* in the laboratory for three subsequent weeks. Data are the percentage of lecithotrophic clutches produced on a given day out of the total number of egg masses deposited by adult specimens (n = 25).

The life-history strategies of planktotrophy and lecithotrophy substantially alter the hatching characteristics and dispersal potential of larvae. Lecithotrophy doubles the encapsulated period of larvae in *Alderia willowi* and reduces fecundity by an order of magnitude (Table 1). However, lecithotrophic larvae are competent to metamorphose even before hatching (Krug 2001, Botello and Krug 2006), reducing their total development time from about 35 days (planktotrophy) to 5 days and presumably decreasing larval mortality. Lecithotrophic veligers are rare in plankton samples above patches of *Vaucheria* spp. (D. Willette and P. Krug, unpubl. obs.), suggesting most metamorphose at or before release. Alternation of developmental modes is thus one dispersal polymorphism, producing long-lived versus short-term larvae in *A. willowi*.

Spontaneous metamorphosis and bet-hedging dispersal strategies in *Alderia willowi*

A second dispersal polymorphism occurs within lecithotrophic clutches of *Alderia willowi*: 0-90% of larvae spontaneously metamorphose prior to emergence or within 2 days of hatching, while their siblings delay settlement until

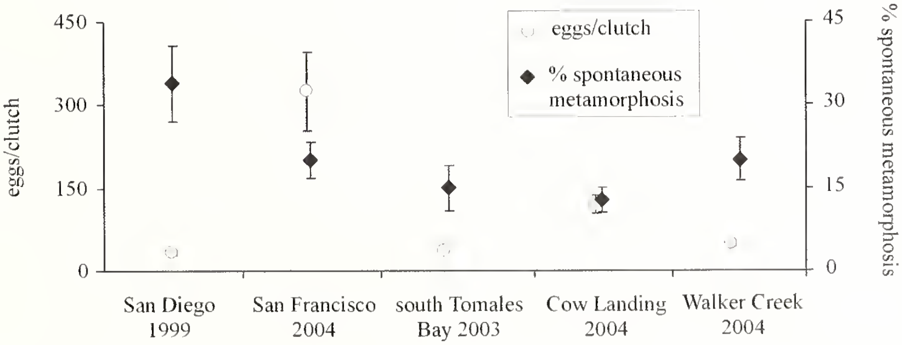

Figure 5. Spontaneous metamorphosis among lecithotrophic larvae of *Alderia willowi* from different years and field sites. Adults were collected from San Diego (August 1999), southern Tomales Bay (September 2003), and from San Francisco Bay, Cow Landing, and Walker Creek (August 2004). Data are percentages of spontaneous metamorphosis (mean ± *SE*) occurring over the first 48 hr post-hatching, in the absence of any inductive substratum.

contacting the host alga (Krug 2001). In many species, larvae are more likely to metamorphose spontaneously with age, a "desperate larva" effect (Gibson 1995, Toonen and Pawlik 2001). The case of *A. willowi* stands in contrast: veligers that do not spontaneously metamorphose die over the next 14 days if denied access to the host, contradicting the hypothesis that non-feeding larvae should lose settlement specificity with age.

Although this trait was highly variable among individuals, the San Diego population hovered around a mean of roughly 25% spontaneous metamorphosis over four years (Krug 2001). Data from four additional populations, surveyed from August to September of 1999, 2003 or 2004, showed a similar trend (Fig. 5). The proportion of spontaneous metamorphosis varied significantly among populations (results of a Kruskal-Wallis test: $H = 11.02$, $P < 0.05$) but was uncorrelated with fecundity (Spearman Rank Correlation: $P > 0.30$). Local conditions were highly variable, as reflected in the mean fecundity of different populations, but good conditions did not produce higher levels of spontaneous metamorphosis (Fig. 5). Stabilizing selection could maintain a mean 15-30% spontaneous metamorphosis at most sites and times, even under optimal conditions. The highest mean value (San Diego, 1999) was due to two slugs that produced clutches with >80% spontaneous metamorphosis, reflecting the variable nature of this trait.

Spontaneous metamorphosis among newly hatched larvae acts as a bet-hedging dispersal strategy, retaining some offspring from each clutch in the parental habitat while allowing the remainder to potentially locate a new algal patch. Bet-hedging is a strategy that raises the geometric mean fitness of a genotype by lowering variation in reproductive success year to year (Seger and Brockmann 1987, Phillipi and Seger 1989, Hopper 1999). A middle-of-the-road approach, bet-hedging genotypes trade the benefit of producing a high recruitment cohort under good conditions against the risk of no reproductive success in bad seasons. This makes it likely that some offspring will survive, regardless of environmental fluctuations. Over many generations, such a genotype has a higher relative growth rate than one that fails to reproduce under any given set of conditions.

In most populations of *Alderia willowi*, about a quarter of lecithotrophic larvae metamorphose with an abbreviated planktonic period or none at all; these larvae are likely to survive if local conditions remain favorable in the natal habitat patch. Their siblings disperse until cued to metamorphose by contact with a new patch of algae, and may survive if conditions in the parental habitat deteriorate. Such a strategy maximizes the chance that some offspring will survive, whether local patches of *Vaucheria* spp. persist or die back. The proportion of spontaneous metamorphosis was also phenotypically plastic, decreasing in response to adult starvation (Krug 2001). Strategies that vary the spatial or temporal distribution of offspring are known for other taxa including plants (Payne and Maun 1981, Telenius and Torstensson 1989, Imbert 1999), mammals (Gaines and McClenaghan 1980), and insects (Harrison 1980, Bradford and Roff 1993, Zera and Denno 1997, Langellotto and Denno 2001), and may be a common evolutionary response to fluctuating environments (Giesel 1976, McPeek and Holt 1992, Kawecki and Stearns 1993, Chia *et al.* 1996).

Effects of developmental pathway on larval swimming and settlement behavior

From a larval biologist's perspective, poecilogony is a chance to explore how conspecific larval morphs produced by divergent developmental pathways compare behaviorally, especially in their response to physical and chemical stimuli during habitat choice. Studies of other poecilogonous species have focused on the ecological effects of different strategies on adult population dynamics (Levin and Huggett 1990, Morgan *et al.* 1999), but not on larval behavior. Variable development in *Alderia willowi* provides the opportunity to compare competent larvae differing greatly in age and in trophic mode.

Krug and Zimmer (2000, 2004) compared physical properties (size, passive sinking rate) as well as swimming behavior for precompetent planktotrophic larvae and both types of competent larvae (Table 2). Quantitative motion analysis revealed the swimming paths of planktotrophic larvae grow straighter and are increasingly directed downwards as larvae mature; competent stages are larger, and sink and swim faster than early stages. When planktotrophic larvae attain competence, they are the same size as 1-day old lecithotrophic larvae, and sink and swim at a similar speed (Table 2). Both competent morphs also share the same shadow response, suggesting behaviors are conserved across developmental programs (Krug and Zimmer 2004). The primary difference in the two morphs is that lecithotrophic larvae swim downward in straighter paths, resulting in a greater net rate of displacement towards the bottom. Modeling efforts indicate differences in swimming behavior could produce a 5-fold greater rate of contact with the bottom for lecithotrophic larvae under natural flow conditions.

If mature planktotrophic larvae tend to encounter dissolved cues while suspended in the water column, stronger behavioral responses might adaptively increase their odds of recruitment. In contrast, because most lecithotrophic larvae hatch in or near suitable juvenile habitat, selective pressure to respond to dissolved cues could be relaxed for this morph. To test these hypotheses, both types of competent larvae were exposed to algal extract or field-collected conditioned seawater (CSW) from within algal patches. Swimming behaviors were quantified through video motion analysis for

Table 2. Physical characteristics and swimming behavior of planktotrophic and lecithotrophic larvae of varying ages in *Alderia willowi* (Krug and Zimmer 2004). Data are mean (± *SE*) larval size, sinking rate, and swimming behvaiors for larvae differing in age and developmental mode. NGD is a ratio of the linear distance from the first to the last point of a given path to the actual distance traveled; a value of zero indicates a circle, whereas a ratio of 1.0 represents a straight line. Vertical speed is a measure of larval movement in the Y-dimension only. Speed is a non-directional scalar, whereas net vertical displacement rate is a vector with negative values indicating downward movement. Behaviors were quantified in the dark using an IR-sensitive video camera.

	Planktotrophic		Lecithotrophic	
	8-d old	32-d old	1-d old	4-d old
Shell size (μm)	126 ± 1	194 ± 1	194 ± 1	194 ± 1
Gravitational fall velocity (mm/s)	−0.90 ± 0.09	−1.59 ± 0.12	−1.52 ± 0.10	−0.99 ± 0.10
Swimming speed (mm/s)	0.85 ± 0.05	1.03 ± 0.09	1.21 ± 0.04	1.32 ± 0.07
NGD ratio	0.83 ± 0.03	0.84 ± 0.02	0.92 ± 0.01	0.90 ± 0.03
Vertical speed (mm/s)	0.42 ± 0.06	0.65 ± 0.07	0.92 ± 0.06	0.99 ± 0.12
Net vertical displacement rate (mm/s)	−0.10 ± 0.08	−0.40 ± 0.12	−0.78 ± 0.09	−0.73 ± 0.21

larvae suspended in the water or moving along the bottom of an experimental chamber. Larvae of both types respond similarly to dissolved cues, turning more frequently and staying close to where the cue is initially perceived (Krug and Zimmer 2000). Larvae suspended off the bottom significantly decrease their speed, swimming in slow helices instead of the straight lines seen in seawater controls. Along the bottom, larvae swim in tight circles or hop, making frequent contact with the substrate (Krug and Zimmer 2000). Such behaviors should increase the chance of contacting a point source of soluble cues (Tamburri *et al.* 1996). Little difference is evident between the two morphs, indicating that selection has maintained a suite of behaviors in relatively non-dispersing larvae that should enhance opportunities for host colonization.

Upon contact with the host alga or after exposure to dissolved cues, larvae attach to the substrate with the propodium (settlement) and begin metamorphosis. Competent larvae of both morphs are equally host specific, settling in response to *Vaucheria longicaulis* but not other macroalgae (Krug and Zimmer 2004). When tested with lecithotrophic larvae, host specificity in *Alderia willowi* is higher than that of any other sacoglossan studied to date, with >90% metamorphosis on *V. longicaulis* but no significant response (0-10%) to 16 alternative algae or mudflat sediments (Krug 2001). Specificity for *V. longicaulis* does not diminish with age (Krug 2001). Such specificity is not found among anaspidean opisthobranchs, which can be stenophagous as adults but settle less specifically as larvae; >30% of *Aplysia californica* larvae metamorphosed on 10 different macroalgae and >25% of *Aplysia oculifera* settled on 4 of 12 tested macroalgae (Pawlik 1989, Plaut *et al.* 1995). Sacoglossans restricted to a single genus or species of host algae often settle specifically on the obligate adult food, reflecting a high degree of coevolution (West *et al.* 1984, Krug 2001, P. Krug, unpubl. obs.).

Dispersal period and rate of metamorphosis

Larvae of *Alderia willowi* vary in their rate of metamorphosis according to age. Larvae of all ages settle within 1-2 hr of exposure to *Vaucheria longicaulis*, but young lecithotrophic larvae (1-2 days post-hatching) delay metamorphosis relative to older larvae (Botello and Krug 2006). About 50% of 1- or 2-day old lecithotrophic larvae complete metamorphosis after 24 hr, but 90-100% response requires 48 hr (Fig. 6). In contrast, 4-day old lecithotrophic larvae metamorphose at an accelerated rate, with >90% of metamorphosis occurring in the first 24 hr of exposure to the host (Fig. 6). Young larvae rarely metamorphose in the first 12 hr after settlement, but a substantial fraction of older lecithotrophic larvae metamorphosed in <12 hr (Botello and Krug 2006). Previously unpublished data show that competent planktotrophic larvae behaved like older lecithotrophic larvae, with most metamorphosis occurring in the first 24 hr (Fig. 6). Thus, in both morphs, longer-lived larvae metamorphose faster after settling on the host.

The increased rate of metamorphosis among older lecithotrophic larvae may be a response to diminishing yolk reserves; 1-2 day old larvae can energetically afford to delay metamorphosis for 12-24 hr while evaluating their surroundings, whereas some older larvae die after 5 days (Krug 2001). The results for competent planktotrophic larvae can be similarly interpreted as an adaptive response, as larvae that have survived a perilous month at sea are under strong pressure to locate a patch of *Vaucheria* without further risk of planktonic mortality. Because competent planktotrophic larvae were fed prior to the assay, they were not energetically constrained as were the 4-d old lecithotrophic larvae; it

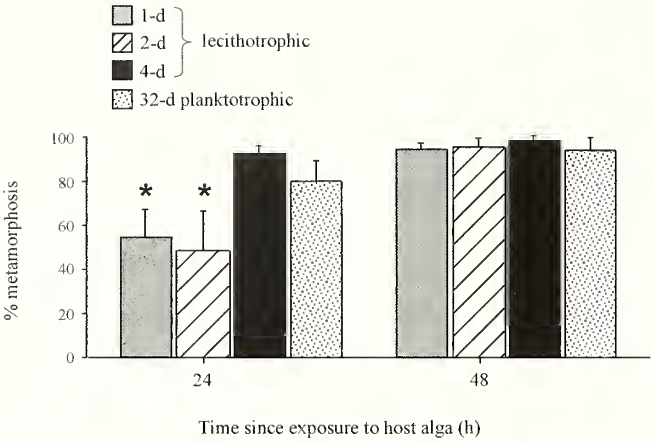

Figure 6. Rate of metamorphosis for different larval morphs of *Alderia willowi*. Cumulative percentages of metamorphosis following exposure to the adult host alga are plotted for lecithotrophic larvae of varying ages (data from Botello and Krug 2006) *vs.* competent, 32-day old planktotrophic larvae. For each age class, percent metamorphosis after 24 *vs.* 48 hr was compared using a Wilcoxon Signed-Rank test to determine if less metamorphosis occurred during the first 24 hr period; *, $P < 0.05$.

therefore appears that metamorphic rate varies according to the life history of a larva, as well as to individual energy levels. Larvae differing widely in age thus have similar patterns of host specificity and settlement behaviors, but differ in rate of metamorphosis depending on an individual's dispersal history.

CONCLUSIONS AND FUTURE DIRECTIONS

The developmental plasticity previously attributed to *Alderia modesta* in fact occurs within its sister species, *Alderia willowi*. Specimens of *A. willowi* can vary the developmental mode of their larvae, alternating seasonally between lecithotrophy and planktotrophy, which is unprecedented among poecilogonous polychaetes and gastropods. Ongoing research aims to unravel the environmental cues that trigger changes in development, which in turn may shed light on the evolutionary forces that favor lecithotrophy in the southern species. Within lecithotrophic clutches of *A. willowi*, a second dispersal polymorphism exists: some larvae spontaneously metamorphose at hatching, while their siblings disperse until stimulated to metamorphose by dissolved or surface-bound carbohydrates from the host alga *Vaucheria longicaulis*. The proportion of spontaneous metamorphosis is highly variable between individuals but the population mean rarely exceeds 25%, even when conditions are optimal. Stabilizing selection might maintain this level to produce a bet-hedging effect, with a quarter of all offspring recruiting into the parental population and the rest dispersing until an algal patch is encountered. The alternative developmental pathways in *Alderia willowi* converge to make a similar competent larva; although differing in age by >30 days, both morphs swim and sink at similar rates, and alter their swimming behavior in response to habitat cues in ways predicted to increase the likelihood of successful recruitment. The rate of metamorphosis, however, changes according to the developmental history and energy level of a given larva.

As biology moves into the proteomic era, poecilogonous species offer the chance to investigate proximal mechanisms such as changes in gene regulation and maternal effects that underlie alternative developmental pathways. Transitions between developmental modes have occurred frequently in many invertebrate taxa, yet stable expression of multiple developmental morphs is vanishingly rare, a paradox waiting to be resolved. The study of poecilogonous species like *Alderia willowi* should provide a more complete understanding of how the interplay between adult and larval ecology shapes adaptive evolution of marine life histories.

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Food intake, growth, and reproduction as affected by day length and food availability in the pond snail *Lymnaea stagnalis*[*]

Andries Ter Maat[1,], Cor Zonneveld[1], J. Arjan G.M. de Visser[2], René F. Jansen[1], Kora Montagne-Wajer[1], and Joris M. Koene[1]**

[1] Faculty of Earth and Life Sciences, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

[2] Laboratory of Genetics, Wageningen University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

Abstract: With the aim of integrating the physiology and evolutionary ecology of *Lymnaea stagnalis* (Linnaeus, 1758), we studied the effects of day length and food availability on the energy budget. Snails were assigned to two different photoperiods and three levels of food availability. The snails were kept individually, and food consumption, growth, and egg production were measured for about 2 months. Snails could nearly compensate for a one-day starvation period by increasing the rate of food-intake. However, food-intake rates did not increase further after a starvation period of 2 days. Growth was well described by the Von Bertalanffy growth equation. The ultimate size of snails kept under medium-day conditions (MD; light:dark = 12:12 h) was not affected by food availability. By contrast, the ultimate size of snails kept under long-day conditions (LD; light:dark = 16:8 h) depended on food availability; those fed the lowest quantities grow the least. Dry-weight densities (dry weight/wet weight) of MD snails were considerably above those of LD snails. In MD snails, food availability did not appreciably affect dry-weight density. By contrast, in LD snails, dry-weight density decreased with decreasing food availability. The reproductive output of LD snails declined with declining food availability, but was 2 to 4 times that of MD snails. The difference in reproductive output was largely accounted for by the difference in stored energy, *i.e.* dry-weight density. To gauge the extent to which the conclusions from our laboratory work applied to free-living snails, a field study was conducted. The wild-caught snails' dry-weight density was also lowest in long-day conditions when most eggs were laid. However, the dry-weight densities during medium and short days were lower than the dry-weight densities of laboratory animals under LD conditions. Thus, in the field, snails stored less energy than in the laboratory.

Key words: allocation, food availability, growth, reproduction

Ecological studies on the energetic costs of growth and reproduction have far-reaching implications for understanding the functioning of animals in relation to their environment (*e.g.*, Dillon 2000). Physiological studies focus on the underlying regulatory processes of growth and reproduction. The latter approach has resulted in a vast knowledge of the basic mechanisms of regulation of growth and reproduction (reviewed in Chase 2002). However, the integration of ecological and physiological knowledge is often hampered because the choice of experimental animal is determined by several considerations that rarely coincide. As a result, few if any experimental animals exist that are thoroughly studied from both perspectives. The aim of the present paper is to fill this gap in knowledge of the physiological ecology of the pond snail *Lymnaea stagnalis* (Linnaeus, 1758).

The great pond snail *Lymnaea stagnalis* is a pulmonate gastropod belonging to the suborder Basommatophora and the family of the Lymnaeidae. This simultaneous hermaphrodite occurs commonly in European lakes, ponds, and ditches and can be easily collected in the field (*e.g.*, Puurtinen *et al.* 2004) where it has an annual life cycle. Eggs are laid in masses containing between 50 and 150 eggs, depending on the individual's body size (Koene *et al.* 2007). Offspring can be produced via self-fertilization and cross-fertilization; when allosperm has been received, there is a preference for outcrossing (Cain 1956, Knott *et al.* 2003). Large populations can also be cultured in the laboratory under semi-natural conditions (Van Der Steen *et al.* 1969). These laboratory conditions allow for extensive control over external factors (*e.g.*, food, temperature, light, etc.) as well as experimental manipulations (*e.g.*, De Visser *et al.* 1994) and neurophysiological experiments (*e.g.*, De Boer *et al.* 1997). As a result, the species has become a widely used, physiological model system for research focusing on neuronal and endocrinological control mechanisms (*e.g.*, El Filali *et al.* 2006; reviewed in Chase 2002).

[**] Current address: Max-Planck-Institut für Ornithologie, Seewiesen, 82319 Seewiesen, Germany, termaat@orn.mpg.de

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The organs and cell groups producing hormones that regulate growth (Geraerts 1976) and reproduction (Geraerts and Algera 1976, Geraerts and Bohlken 1976, De Boer *et al.* 1997) are known, as well as the amino acid sequences of these hormones (Ebberink *et al.* 1985, Vreugdenhil *et al.* 1985, Smit *et al.* 1988, De Lange *et al.* 1997, Jiménez *et al.* 2004). For example, growth is regulated by a growth hormone produced by the light green cells (LGC). Egg laying is triggered by the discharge of the neurosecretory caudodorsal cells (CDC; Ter Maat *et al.* 1989). These neurons are electrically coupled and during a discharge, a massive amount of the egg-laying hormone is released. This hormone, called CDCH, has been fully characterized (Geraerts *et al.* 1985, Jiménez *et al.* 2004) and gives rise to ovulation, which results within two hours in an egg mass being oviposited.

Despite the detailed knowledge about the neuroendocrinological regulation of growth and reproduction in this species, surprisingly little is known about the functioning of these processes in relation to the animal's environment. Environmental variables, like day length and food availability, are known to affect the allocation of energy to growth and reproduction (*e.g.*, Scheerboom 1978, Bohlken and Joosse 1982). However, no detailed studies have been performed to quantify these environmental factors. Such studies should be quite relevant for an understanding of the hormonal regulation of growth and reproduction. To bring these two fields of research closer together, in the present study we focused on the interaction between photoperiod and food availability in the allocation of energy to growth and reproduction.

MATERIALS AND METHODS

Experimental design

Two laboratory experiments were performed, one under medium-day conditions (MD: light:dark = 12:12 h; duration 80 days), the other under long-day conditions (LD: light: dark = 16:8 h; duration 57 days). The adult snails, taken from a mass culture bred under standard conditions, *i.e.*, MD (Van der Steen *et al.* 1969), were kept individually in perforated polyethylene beakers with a lid (460 ml). At the start of the experiment, the snails were adult. The shell lengths of the MD animals were 24.40 ± 1.55 mm, the LD animals 22.71 ± 0.91 mm. Group size was 20 in the MD experiment and 15 in the LD experiment. The perforated beakers were placed in a tank with continuous water exchange using Amsterdam tap water through Cu-free piping. The water temperature was kept at 17.5 ± 0.5 °C. Beakers were changed every 3 or 4 weeks. Coprophagy was not prevented.

In both experiments, three levels of food availability were studied by varying the frequency at which food was supplied. The snails in group 1 received lettuce in excess of their requirements every day, those in group 2 at two subsequent days followed by one day of starvation, and those in group 3 every third day followed by two days of starvation.

Measurements of food consumption, growth, and egg production were made. A broad-leaved variety of lettuce was used as food. From the flat parts of the leaves, where only small vascular bundles are present, circular discs were punched with a surface area of 19.6 cm^2. Snails were either provided with 2 discs or starved. After 24 h the remaining lettuce was removed from the jars, spread out on a Perspex plate, and covered with a glass plate. The plates were subsequently recorded on a video tape, and recordings were digitized to determine the surface area. The difference between the area provided and remaining was used as a measure for consumption.

Every two weeks the shell heights were measured with a caliper to the nearest 0.1 mm. At the end of the experiment, snails were frozen in liquid nitrogen. After thawing, the shell was separated from the body, and the wet weight of the body was determined. The shell and body were freeze-dried, after which they were weighed to the nearest 0.1 mg. Dry weight density (*i.e.*, the ratio of dry weight to wet weight, expressed as a percentage) was used as a measure of consumption.

Egg masses were collected every day. The egg masses were stored in 70% ethanol until the number of eggs per egg mass was counted.

Data analysis

Von Bertalanffy growth curves were fitted to the data on shell heights for individual snails. The Von Bertalanffy growth curve is given by the equation:

$$h(t) = h_e - (h_b - h_e)\exp\{-gt\} \qquad (1)$$

where h(t) denotes the current shell height; h_e, the ultimate shell height, which may eventually be reached if the snail is kept under constant conditions for a long period; h_b, the shell height at the start of the experiment; and g, the Von Bertalanffy growth coefficient. The use of the Von Bertalanffy curve has previously been shown to be appropriate for this species (Zonneveld and Kooijman 1989).

For regression analyses we assumed a normally distributed scatter with homogeneous variance around the model curves. Given this assumption, maximum likelihood estimates are given by the least squares method. To obtain the least squares estimates of the parameters, we used the Gauss-Newton method (Richter and Sondgerath 1990). Standard deviations of the parameters were estimated according to the large sample theory of maximum likelihood estimators (Cox and Hinkley 1974). Comparisons between groups were made using Tukey's post-hoc test.

Field study

For nearly two years, from 29 August 2002 to 14 April 2004 we collected a total of 564 *Lymnaea stagnalis* specimens from two ditches in the Eempolder near Amsterdam, the Netherlands. The Eempolder is a protected landscape enclosed by dikes consisting of pastures separated by ditches. Samples were taken in all months of the year, and on each sampling date we tried to collect a representative sample of both adults and juveniles. Immediately after collection, the animals were weighed and shell length was measured. The snails were subsequently dissected and the shell, albumen gland, and prostate gland were removed and weighed (these data will be published elsewhere, Koene *et al.*, unpubl. data). The soft body parts were freeze dried and weighed. All animals were checked for parasites. All year round, almost 50% of the snails collected in the field are infected with one or more species of parasites, among which were *Trichobilharzia ocellata, Echinostoma revolutum, Opisthioglyphe ranae, Hypoderaeum conoidum, Diplostomum spathaceum*, and *Pseudoechinoparyphium echinatum* (Loy and Haas 2001, De Jong-Brink and Koene 2005, Koene *et al.*, unpubl. data). In the current paper we present data on the dry weight density of individuals not containing parasites ($N = 283$).

RESULTS

Growth and food availability

Von Bertalanffy growth curves were fitted to the measured shell heights (Table 1). The growth rate parameter g in equation 1 is a measure for the curvature of the growth curve. If the time constant g^{-1} is larger than the duration of the experiment, the curvature will be barely observable; hence, it can be very difficult to estimate this parameter. This situation applied to only 7 MD snails of group 3, which were the slowest growing snails. Standard deviations are based on the estimates of the parameters for the individual snails.

Table 1. Means (and standard deviations) of parameter estimates of Von Bertalanffy growth curves. Abbreviations: h_e, ultimate shell height; h_b, initial shell height; g, the Von Bertalanffy growth coefficient; *n*, sample size.

Day length	Food regimen	h_e (SD) cm	h_b (SD) cm	g (SD) d^{-1}	*n*
MD	1	3.45 (0.22)	2.25 (0.20)	0.0394 (0.018)	20
	2	3.53 (0.28)	2.25 (0.17)	0.0284 (0.0077)	20
	3	3.43 (0.53)	2.25 (0.18)	0.0145 (0.0050)	13
LD	1	3.33 (0.29)	1.86 (0.12)	0.0493 (0.014)	15
	2	3.35 (0.27)	1.99 (0.084)	0.0321 (0.010)	15
	3	2.62 (0.18)	2.03 (0.12)	0.0307 (0.011)	15

The growth coefficient g was significantly affected by day length and food availability (two way ANOVA; day length: $F = 15.6$, $df = 1, 92$, $P < 0.001$; food availability: $F = 25.4$, $df = 2, 92$, $P < 0.001$), but there seemed to be no interaction between these two factors ($F = 2.0$, $df = 1, 92$, $P = 0.14$). The growth coefficient was larger in LD snails than in MD snails, indicating that the LD animals grew faster. The growth coefficient decreased with decreasing food availability. In LD conditions, the animals had slower growth. Also, limited food supply led to slower growth rates.

Food availability had no effect on the ultimate length in MD snails (one way ANOVA, $F = 0.28$, $df = 2, 50$, $P > 0.5$). In LD snails, food availability also had no effect on the ultimate lengths of groups 1 and 2, but snails in group 3 remained much smaller ($P < 0.001$). The ultimate shell heights of groups 1, 2, and 3 of MD snails differed slightly from those of group 1 and 2 of LD snails (one way ANOVA, $F = 5.6$, $df = 1, 68$, $0.01 < P < 0.05$). In conclusion, when food was abundant, day length had a small effect on the ultimate size attained, even though this length was reached later by LD animals. However, when food was limited, LD animals grew more slowly and reached a smaller ultimate size.

Dry weights consist of structural body mass and stored energy. The dry weight density, *i.e.*, dry weight per wet weight (without shell), can be used to compare the amounts of stored energy in different groups. Dry-weight densities of MD and LD snails at the three levels of food availability are shown (Fig. 1A). A two-way ANOVA showed that MD and LD snails reacted similarly to food availability (interaction between day length and food availability: $F = 2.3$, $df = 2, 99$, $P > 0.05$). The MD snails had higher dry-weight densities than LD snails ($F = 293.0$, $df = 1, 99$, $P < 0.0001$). There was a significant overall effect of food availability ($F = 4.05$, $df = 2, 99$, $P < 0.05$); the lowest dry-weight densities occurred in the animals receiving the least amount of food and *vice versa* (Tukey at $P = 0.05$).

In summary, food availability determines only growth rate and not ultimate size in medium-day animals; however, under long-day length conditions, food availability was a major determinant of final size as well as growth rate. Stored energy was higher with higher food availability in both groups.

Food consumption and fecundity

At each level of food availability, the fecundity of LD animals was higher than that of MD animals. Also, when food was present on two out of three days, fewer eggs were laid on the day when no food was present. However, when

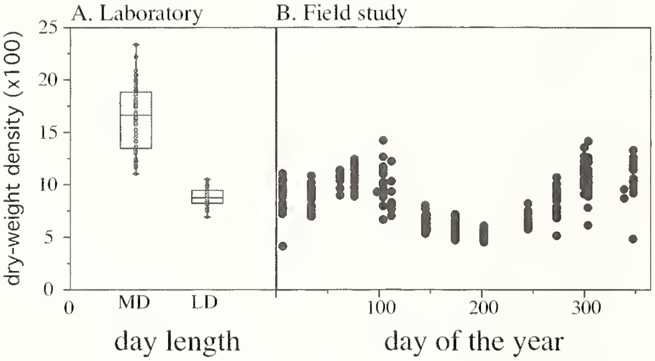

Figure 1. Dry weight densities under LD (Long Day, 16:8 h light: dark) and MD (Medium Day, 12:12 h light:dark) conditions in the laboratory and in the field. A. Laboratory snails. Data are shown for animals that received food on all days of the schedule. LD animals had lower dry weight densities than did MD animals ($P < 0.001$; Student's *t*-test). B. Dry weight densities of wild-caught snails. During the year, a minimum was reached during summer.

food was provided on one day out of three, egg production was equal (MD) or lower (LD) than on the days the animals went without food. Table 2 provides the data on consumption and oviposition on each of the three days of the food cycle. The pattern of egg production was established during the first three-day cycle and persisted throughout the experiment. In the MD experiment, 19, 17, and 10 snails of group 1, 2, and 3, respectively, produced at least 1 egg mass. In the LD experiment, all of the snails produced egg masses. The average interval between oviposition bouts depended on food availability. In LD animals, the mean intervals were 2.13 (SD = 0.26), 2.41 (SD = 0.36), and 4.18 (SD = 0.89) days for groups 1, 2, and 3, respectively. In MD animals the intervals were 9.85 (SD = 9.66), 16.77 (SD = 6.49), and 36.89 (SD = 26.35). Animals that did not lay any eggs were excluded from the analyses; this occurred only in the MD group as follows: group 1, $n = 1$ snail; group 2: $n = 2$; group 3: $n = 10$. The differences between food regimes were significant in both LD (Kruskal-Wallis, $H = 31.6$, $df = 2$, $P < 0.0001$) and MD conditions ($H = 13.7$, $df = 2$, $P < 0.001$). LD animals at all three levels of food availability laid more eggs and egg masses than their MD counterparts. These results are in keeping with earlier studies on the effects of daylength (Bohlken and Joosse 1982).

Egg masses shown in Table 2 for a certain day were collected at the end of that day of the food cycle. Snails that were fed every day (*i.e.*, group 1) showed no preference for any day of the food cycle to oviposit (MD: Chi-square, $\chi^2 = 1.46$, $P > 0.01$; LD $\chi^2 = 0.536$, $P > 0.01$). Snails of group 2 laid the fewest egg masses on the day they were starved. However, MD snails laid the most egg masses on the second day of the food cycle ($\chi^2 = 45.2$, $P < 0.01$), whereas LD snails laid the most egg masses on the first day ($\chi^2 = 57.1$, $P < 0.01$). No preference could be demonstrated for MD snails of group 3 ($\chi^2 = 1.75$, $P < 0.01$), probably due to the small number of egg masses that were laid. In LD group 3 snails, most egg masses were laid on the days they were starved ($\chi^2 = 9.66$, $P < 0.01$).

Because the data in Table 2 are not independent, the results should be interpreted with caution. Nevertheless, we think that the differences between the groups can be attributed to the food availability per se. In both groups that were fed every day there was not even a slight indication of a periodicity, whereas in all other groups the periodicity was pronounced, with the exception of MD group 3, where very few egg masses were laid. The patterns of egg laying during the three-day cycle did not change during the experiment.

Table 2. Lettuce consumption, egg mass, and egg production on each day of the feeding protocol. Data for MD (Medium Day) and LD (Long Day) animals. Means and standard deviations are given. There were three feeding schedules: 1, food every day; 2, food on days 1 and 2; 3, food on day 1 only. The data from the days the animals went without food are in italics.

Lettuce consumption (cm^2 per day per animal)

Day length	Food regimen	Day 1	Day 2	Day 3
MD	1	14.94 (3.27)	14.50 (3.45)	13.79 (3.39)
	2	23.71 (3.44)	17.86 (3.10)	*0.00 (0.00)*
	3	21.12 (4.09)	*0.00 (0.00)*	*0.00 (0.00)*
LD	1	16.35 (3.20)	16.14 (4.06)	16.09 (2.58)
	2	22.99 (5.18)	18.07 (5.02)	*0.00 (0.00)*
	3	14.47 (2.76)	*0.00 (0.00)*	*0.00 (0.00)*

Eggs per day per animal

Day length	Food regimen	Day 1	Day 2	Day 3
MD	1	13.62 (7.79)	15.39 (11.42)	16.31 (12.53)
	2	10.51 (9.35)	17.23 (12.31)	*4.33 (5.99)*
	3	1.53 (2.65)	*2.59 (3.86)*	*1.42 (3.17)*
LD	1	41.29 (27.14)	37.77 (17.61)	38.86 (18.50)
	2	36.79 (14.75)	27.05 (11.8)	*7.49 (6.85)*
	3	8.82 (6.53)	*15.16 (8.07)*	*15.50 (8.94)*

Egg masses per day per animal

Day length	Food regimen	Day 1	Day 2	Day 3
MD	1	0.15 (0.08)	0.17 (0.10)	0.18 (0.12)
	2	0.11 (0.09)	0.18 (0.11)	*0.04 (0.05)*
	3	0.02 (0.03)	*0.03 (0.04)*	*0.01 (0.03)*
LD	1	0.50 (0.22)	0.47 (0.14)	0.46 (0.21)
	2	0.59 (0.17)	0.49 (0.18)	*0.19 (0.13)*
	3	0.18 (0.15)	*0.27 (0.13)*	*0.30 (0.15)*

The relationship between egg production and consumption is shown (Fig 2). In groups 1 and 2, the slope of the fit was 2.247 and 2.211 eggs × cm^2 lettuce. In group 3, many animals did not lay eggs and there was no correlation between consumption and egg production. Combining all three groups yielded a slope of 1.419. In LD animals, all of which laid eggs, the slope of overall fit was 2.242 eggs per cm^2 lettuce. We conclude that an egg yield of about 2.2 eggs per cm^2 lettuce is a reasonable estimate.

Dry weight density in the field

Adults were present throughout the year and two generations partially overlap during the spring. Dry-weight densities were determined for 283 unparasitized specimens of *Lymnaea stagnalis* collected over the course of the year (Fig. 1). Dry-weight density varied during the year and was lower in summer, the season when most eggs are produced. The overall level of dry-weight density was lower in the field than in the laboratory. This was true for both long and short-day length conditions.

DISCUSSION

Food consumption

After starvation, individuals of *Lymnaea stagnalis* had higher consumption rates than snails that were fed continuously. Thus snails of group 2 appeared able to compensate for the day they were starved. This explains the relatively slight differences in growth and reproduction.

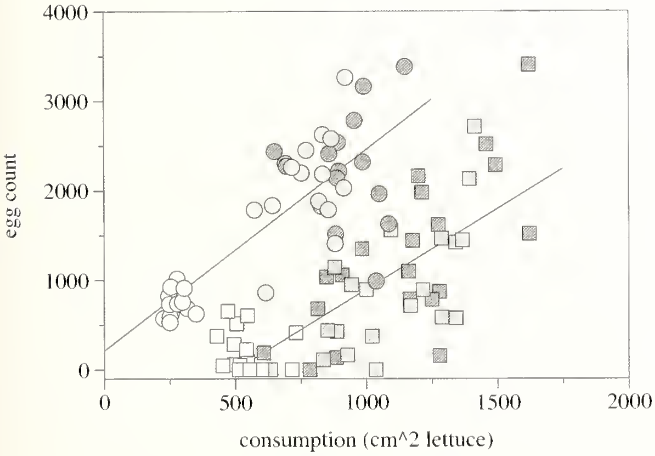

Figure 2. Relationship between food consumption and egg production in snails reared under LD (Long Day, circles) and MD (Medium Day, squares) conditions with the three feeding cycles. White fill, regimen 3; intermediate fill, regimen 2; dark fill, regimen 1. The lines are fitted to all the data of the MD and LD groups, respectively.

Growth

Bohlken and Joosse (1982) also studied the effects of day length on growth and reproduction in *Lymnaea stagnalis.* They reared a few hundred snails in one large tank under the same LD and MD conditions we used. We fitted growth curves to the data of Bohlken and Joosse (1982), yielding the following parameter estimates: for MD snails, $h_e = 3.54$ cm, $g = 0.014\ \mathrm{d}^{-1}$; for LD snails, $h_e = 2.97$ cm, $g = 0.017\ \mathrm{d}^{-1}$. The estimate for the growth rate parameter of MD snails corresponds to the one we determined for group 3 snails. The ultimate shell height for MD snails is equal to the one we found in the present experiment for the three groups; for LD snails it is between that of groups 2 and 3. This comparison suggests that in the experiment of Bohlken and Joosse (1982), food consumption was as limited as in our group 3. Data on the growth of snails that were kept individually and fed a limited amount of lettuce are provided by Geraerts (1976) and Bohlken *et al.* (1984). Growth curves were fitted to their data on control snails. In both cases, values of the growth rate parameter are in agreement with those found in the present study for the best-fed snails ($0.04 <$ growth rate < 0.06). Also, they were much higher than those reported by Bohlken and Joosse (1982). Apparently, snails kept in isolation have much better feeding conditions than do snails kept in large groups.

We found no differences between the ultimate shell heights of groups 1, 2, and 3 of MD snails. Thus the maintenance costs in these groups should eventually be identical. Because the rate of food intake was not identical, the less fed groups must allocate less energy to reproduction. Indeed, MD snails of group 2 produced fewer eggs than those of group 1, while the size differences between the snails were small throughout the experiment.

According to Kooijman (1993), the Von Bertalanffy growth coefficient decreases with increasing maximum storage capacity, because the animal has to build up the energy stores. The larger these stores, the longer it takes to build them up. LD snails had lower dry-weight densities than MD snails, so in all likelihood LD snails had less stored energy. In accordance with this prediction, LD snails had the higher Von Bertalanffy growth rates.

Input from the tentacles on the LGC, which produce a growth hormone related to insulin, may provide one way in which environmental factors could influence growth (Roubos and Van der Wal-Divendal 1982, Smit *et al.* 1988).

Timing of oviposition in relation to the food cycle

In both MD and LD snails, the timing of oviposition seemed to depend on the availability of food. However, MD and LD snails reacted differently to the presence or absence of food. Both LD and MD snails suppressed egg laying when food was absent. Under LD conditions, egg-laying of group 2 was maximal on the first day after starvation. Under MD conditions, egg-laying in group 2 was maximal on the second day after starvation. A comparison with group 3 is not feasible because so few snails laid eggs. However, the fact that in LD snails in group 3 egg-laying was maximal on the second day of starvation is remarkable. Because snails of group 3 were starved for two days, the reduced egg laying on the day that the snails were fed might reflect a time-budget problem: the snails had no time to oviposit since they were busy feeding. However, this suggestion is not supported by the following observation. Snails of group 2 laid most egg masses on one of the days they were fed, whereas LD snails of group 3 laid most egg masses on one of the days they were starved. Yet the food-intake rates of groups 2 and 3 were equal. Hence we conclude that there was no time-budget conflict between egg-laying and feeding during the experiment.

A number of factors are known to affect the excitability of the egg-laying, hormone-producing CDC's, and hence egg laying. A discharge of this cell cluster is an all-or-nothing phenomenon; between discharges, the CDC remain electrically silent. One trigger for egg laying is a transfer from dirty to clean water (Clean Water Stimulus: Ter Maat *et al.* 1983). The present experiments suggest that food availability also affects the excitability of the CDC's, although indirectly, which is in agreement with electrophysiological findings (Ter Maat *et al.* 1982). Reduced food availability does not affect the size of the egg mass but does cause an increase of the interval between the deposition of successive egg masses. Before oviposition, energy allocated to reproduction is stored in various glands, including the albumen gland. The fact that both day length and food availability affect the reproductive rate but not the size of an egg mass suggests that oviposition is likely to occur if the gland's contents pass a certain threshold. This suggests that the filling of the albumen gland (and/or other glands) influences the excitability of the CDC's, possibly via the activation of stretch receptors. This idea is supported by the finding that in *Lymnaea stagnalis* albumen glands are heavier when the animals go longer without egg-laying (Koene and Ter Maat 2004). In contrast, in the garden snail *Helix aspersa* (Müller, 1774) the number of ripe oocytes in the ovotestis provides a permissive signal for the occurrence of egg-laying (Antkowiak and Chase 2003). We think that in *L. stagnalis*, storage of packaging material for the eggs is the critical factor in egg laying. Given these findings, it would be interesting to study whether egg-laying and the excitability of the CDCs depends on sensory signals from the albumen gland or from other accessory organs containing packaging material.

Comparison of MD and LD snails

Average somatic and reproductive production rate was a function of the average rate of food intake. Although such relationships are difficult to interpret, there was a clear difference between MD and LD snails. With regard to somatic production, however, there is no difference. There was a very clear difference in the reproductive output of MD and LD snails. At the same average food-intake rate, LD snails produced about 20 eggs per day more than MD snails. Qualitatively, this agrees with the observation that the daily egg production rate in LD snails was about 2 to 3 times that of MD snails.

If somatic production was more or less the same in LD and MD snails, how were LD snails able to maintain a much higher reproductive rate? LD snails had lower dry weights and therefore probably fewer energy reserves. The difference in dry weight acquired during the experiment for MD and LD snails, as calculated from the dry weight density and the volume increase, was about 0.12 g. The dry weight of eggs, including the capsule in which the eggs are embedded, is about 0.15 mg per egg (Zonneveld and Kooijman 1989). Hence the difference in acquired dry weight is equivalent to about 800 eggs. The experiment lasted for approximately 60 days, during which LD snails produced on the average 20 eggs per day more than did MD snails (1200 eggs more during the experiment). The difference in dry weight explains about 70% of this difference in egg production. Thus, we conclude that the difference in the rate of egg production was largely due to a decrease in energy reserves of LD snails compared to MD snails.

In the field, *Lymnaea stagnalis* is essentially an annual species, breeding in the summer season. Light conditions similar to our MD treatment are experienced in autumn and spring, during the juvenile period. It is advantageous to have large energy stores during this period, because food availability will be low and unpredictable. Light conditions similar to our LD treatment are experienced in the field in late spring and in the summer. Food availability will be predictably high in this period so there should be no need to have large energy stores. To maximize the reproductive output, the energy stores should be depleted. Our experiments were performed at a constant day length, while in the field, day length gradually increases to LD conditions with the onset of the summer. Hemminga *et al.* (1985) showed that snails indeed draw on their energy reserves after a change from a short to a long day length.

In conclusion, the current study shows that the high rate of reproduction under long day conditions can be maintained by keeping stores at a low level. In contrast, medium day animals invest more in storage, and lay eggs only when energy storage is above a certain level. The data on size and dry weight density in the field show a similar relationship with day length as the laboratory data, in that long days are associated with high fecundity and low energy storage. However, a major difference between field and laboratory data is that dry weight densities are higher in the laboratory, presumably due to differences in feeding conditions.

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Phally polymorphism and reproductive biology in *Ariolimax (Ariolimax) buttoni* (Pilsbry and Vanatta, 1896) (Stylommatophora: Arionidae)*

Janet L. Leonard[1], **Jane A. Westfall**[2], **and John S. Pearse**[1]

[1] Joseph M. Long Marine Laboratory, University of California-Santa Cruz, Santa Cruz, California, 95060, U.S.A., jlleonar@ucsc.edu

[2] Department of Anatomy and Physiology, Kansas State University, Manhattan, Kansas 66506-5802, U.S.A.

Abstract: Phally polymorphism, whereby some individuals in a hermaphroditic species have a complete functional penis (euphally), whereas others lack a penis (aphally) or have a reduced, non-functional penis (hemiphally), has evolved many times in pulmonate gastropods. Since the discovery of apophallation (penis amputation) in *Ariolimax* Mörch, 1860, aphallates in this genus have been attributed to apophallation. In laboratory studies in *Ariolimax (Ariolimax) buttoni* (Pilsbry and Vanatta, 1896), we found aphally in juveniles as well as in individuals reared in isolation to adulthood, demonstrating that the aphallate condition is not always due to apophallation. Some aphallate individuals reared in isolation from hatching produced eggs and viable hatchlings, providing the first demonstration of uniparental reproduction in this species. Egg-to-egg generation time in laboratory-reared individuals ranged from 10 months to more than 24 months. Anatomical data also elucidate the reproductive cycle of this species. Four reproductive states have been identified by the appearance of the reproductive system. Spring and early summer populations consist of individuals in the immature and intermediate reproductive states. The hypertrophied state was found from autumn until early spring. Egg-laying occurred in the laboratory in the fall and winter. Copulation consists of unilateral or simultaneously reciprocal intromissions and occurred in the laboratory between February and September. Very long copulations (more than 7 h) are more frequent than in other species of *Ariolimax*. Phally polymorphism, uniparental reproduction, and the variation in generation time should play important roles in determining the variance of mating success and the potential for sexual selection in this hermaphroditic species.

Key words: gastropod, genitalia, aphally, life history, self-fertilization

Banana slugs, giant stylommatophoran slugs of the genus *Ariolimax* Mörch, 1860, are common and conspicuous members of temperate rain forests and other mesic habitats of the northwestern coast of North America. Although banana slugs are well-known and popular with the general public, remarkably little is known about their biology (but see Harper 1988, Leonard *et al.* 2002, Cody 2006, Pearson *et al.* 2006). In *Ariolimax,* as in stylommatophorans in general, taxonomy has been based on genital characters (Pilsbry 1948). Eberhard (1985) suggested that where genital characters have evolved rapidly enough to distinguish species (and even subspecies), sexual selection has played an important role in the evolution of the group. The genus *Ariolimax* offers a particularly good opportunity to test this hypothesis since it consists of a small number of taxa (Roth and Sadeghian 2003, Pearse *et al.* 2007), most of which are found in coastal Central California in quite similar habitats, but which have very divergent genitalia and sexual behavior (Leonard *et al.* 2002, 2005). Accurate information about the reproductive biology of *Ariolimax* spp. is necessary to perform such a test.

One of the most mysterious aspects of *Ariolimax* biology is the existence of aphallate individuals and the extent to which these can be explained by apophallation. Apophallation is a behavior first observed in *Ariolimax (Meadarion) californicus* (Cooper, 1872) (Heath 1916, Leonard *et al.* 2002) and subsequently in *Ariolimax (Meadarion) dolichophallus* Mead, 1943 (Mead 1942, 1943, Harper 1988, Leonard *et al.* 2002) whereby the penis is sometimes chewed off at the end of copulation in these simultaneous hermaphrodites. Since Heath (1916) most authors have been content to explain aphallate individuals of *Ariolimax* as the product of apophallation. However, Heath himself expressed doubt, saying, "years ago I visited Hog Island in Tomales Bay, and found over 400 specimens ... every one of the specimens was totally lacking a penis or any sign of one." (quoted in Mead, 1943, p. 685). Mead (1943) later collected in the same area, finding no slugs at Hog Island and an entirely phallate population nearby. Paull (1951) examined the gross anatomy of *Ariolimax buttoni* (Pilsbry and Vanatta, 1896) (= *A. columbianus* (Gould in Binny, 1951), see below) at Mills College in Oakland, California and found that of the 27 adult specimens she dissected, none had a penis. More recently, Roth (2004) reviewed anatomical descriptions of *Ariolimax* in detail, and suggested that the descriptions are inconsistent with what would be expected if a penis were amputated during copulation. He argued that apophallation should sever both the penis and vas deferens, leaving unconnected remnants of both and an intact penis retractor muscle, whereas Pilsbry and Vanatta's (1896) drawing of *Aphallarion buttoni* showed an intact vas deferens connected to the genital atrium by a short bulb with no penis and no penis retractor muscle. Roth (2004) concluded that the descriptions were more consistent with a phally polymorphism. Such phally polymorphisms are well-known and widespread in pulmonates, including stylommatophorans (Tompa 1984, Pokryszko 1987, Baur and Chen 1993, Doums and Jarne 1996, Viard *et al.* 1997, Doums *et al.* 1998, Gómez 2001), and there are even instances of a phally polymorphism having lead to an erroneous splitting of a single species into two genera (*e.g.*, Lace 1992).

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Here, we present previously unpublished observations on aphallate and euphallate individuals in two populations of *Ariolimax (Ariolimax) buttoni* and data showing aphally in individuals which cannot have been involved in apophallation because they were reared in isolation to adulthood. These observations demonstrate that this species has a phally polymorphism, with both aphallate and euphallate individuals in some populations. We also present laboratory observations on the sexual behavior and life cycle of *A. buttoni* and document uniparental reproduction in this species.

MATERIALS AND METHODS

Taxonomy

The animals used in this study are *Ariolimax (Ariolimax) buttoni*. This species was first described by Pilsbry and Vanatta (1896) from a large series of aphallic ariolimacines from Oakland, California as a new genus. Subsequently, this taxon was synonymized to *Ariolimax (Ariolimax) columbianus* (Gould in Binny, 1951) (Waste 1940, Mead 1943, Pilsbry 1948) in the expectation that the aphally could be attributed to apophallation during copulation. This species was considered to be the only ariolimacine to include maculate individuals and was stated to have a range extending from Tuolomne County, Monterey County, the eastern shore of San Francisco Bay, and the city of San Francisco in California, north to southeastern Alaska (Pilsbry 1948, Roth and Sadeghian 2003). Recent molecular evidence suggests that *A. columbianus*, as defined by Mead (1943) and Pilsbry (1948), is not monophyletic but rather that populations of *Ariolimax* north of Mendocino County, California (the true *A. columbianus*, since the species was described from specimens collected near the Columbia River, see discussion in Pilsbry 1948) are evolutionarily distinct from the more southern populations (Leonard *et al.* 2005, Pearse *et al.* 2005). The name *Ariolimax buttoni* (Pilsbry & Vanatta, 1896) has been revived to designate the southern clades formerly included in *A. columbianus* (Pearse *et al.* 2007). *Ariolimax buttoni* and *A. columbianus* do not even represent sister clades (Pearse *et al.* 2007). Like *A. columbianus*, *A. buttoni* may be either maculate or immaculate. *Ariolimax (Meadarion) brachyphallus* Mead, 1943 is sympatric with *A. buttoni* in San Francisco but all individuals used in this study from San Francisco have been identified by molecular markers as belonging to *A. buttoni*. There are no reports of sympatric ariolimacines in Alameda, Sacramento, Mendocino, or Marin counties.

Animals

Mills College animals

Specimens were collected from March 1951 to February 1952 in two locations; the Mills College campus (37° 46' 41"N, 122° 10' 49"W) and the nearby Leona Heights Park (37° 47' 31"N, 122° 10' 41"W) in Oakland, Alameda County, California. Approximately 24 animals from each location were housed in the laboratory in terraria filled with damp earth and fed with lettuce. Other animals were dissected shortly after collection.

UCSC animals

Specimens were collected from five locations in Central California: (i) a wooded area of Mount Parnassus on the campus of the University of California, San Francisco, San Francisco County (37°45' 38"N; 122° 27' 28"W); (ii) a levee of Staten Island in the Cosumnes River, Sacramento County (38° 15' 56"N, 121° 26' 31"W); (iii) near Comptche, Mendocino County (39° 15' 54" N, 122° 35' 24" W); and two locations in Marin County, namely (iv) near Muir Woods in central Marin County (37° 53' 07"N, 122° 32' 26"W) and (v) the west side of Tomales Bay (38° 10' 25"N, 122° 55' 26"W) in western Marin County. Animals were maintained in the laboratory as groups or individuals in plastic boxes as described elsewhere (Leonard *et al.* 2002).

Anatomical studies

Mills College animals

A total of 67 individuals of *Ariolimax buttoni*, ranging from 16 to 55 grams in weight, were dissected. Three slugs were dissected at the beginning and middle of each month from March to May 1951. Five slugs were dissected every two weeks from October 1951 to February 1952. Two of the slugs were taken from the animals kept in terraria whereas the other three were collected from the field. The day before dissection, the slugs were weighed and the next day they were drowned, dried to remove excess mucus, pinned to a dissecting tray and dissected in water. A longitudinal incision was made on the right side from the caudal pore to the mouth, the dorsal skin retracted, and the genitalia freed by severing connective tissue and the protractor muscles of the tentacles. After observation of the intact condition, the skin was cut around the atrium and the reproductive system was laid out separately for measurement. The data recorded were: a) the color and degree of development of the reproductive system; b) the position of the vas deferens and the presence or absence of a penis and/or terminal bulb on the vas deferens; c) the approx. width of the vagina and oviduct; d) the approx. length and width of the spermatheca; e) the approx. length and width of the ovotestis; and f) the approx. length of the albumen gland.

UCSC Animals

Anatomical studies were conducted with *Ariolimax buttoni* derived from two populations (Staten Island and Tomales Bay) and reared as described by Leonard *et al.* (2002). Three of the individuals were collected from the field and then maintained in the laboratory until their deaths, at which time they were frozen. The other individuals were hatched in the laboratory and frozen when slightly over 30 months of age. They were thawed just before dissection, pinned to a dissecting tray, and dissected under water. An incision was made through the body wall with scissors along the left side, just above the foot, and the dorsal body wall peeled back to expose the digestive system and genital organs, and the albumen gland, ovotestes, and male and female parts of the genital organs teased apart. The condition of the spermatotheca and the presence or absence of the penis was noted. If absent, the presence or absence of a penis stub was noted as well as the course of the vas deferens. The male and female portions of the genital organs were sketched for most specimens; in a few cases they were measured and photographed through the dissecting microscope, using an Olympus Camedia C-3040 digital camera. Upon completion, the dissected animals were preserved and archived in 10% formalin.

Rearing studies

Two sets of rearing studies have been conducted with *Ariolimax buttoni.* The first series was conducted at Mills College (Mills College animals) from 1951 to 1952 (Westfall 1960). Eggs and juveniles were maintained in terraria filled with damp earth along with the parents. They were fed lettuce leaves. In total 17 juveniles ranging in age from 1 day to 19 weeks were taken for anatomical study.

The second rearing study was conducted at the Long Marine Laboratory of the University of California-Santa Cruz (UCSC animals) from 2003 to 2006. Slugs were collected from Staten Island and Tomales Bay and held in group boxes. Eggs laid in fall and early winter 2003-2004 were maintained as described previously (Leonard *et al.* 2002) and hatchlings weighed when found and transferred to individual plastic containers. All containers were cleaned and lettuce added weekly. After the juveniles reached 5 g in weight, dry cat food was also given. Slugs were transferred to larger containers as necessary as they grew. They were weighed weekly until 6 mo and biweekly thereafter.

All hatchlings were reared in isolation until June 2004 when, as part of a mating study (Leonard, *et al.* unpubl. obs.), 40 individuals from the Staten Island group and 40 from the Tomales Bay group were randomly assigned to a mating treatment. Eight individuals, each from Staten Island and Tomales Bay parents, were assigned to the single animal (continued isolation) treatment. Equal numbers of individuals from Tomales Bay and Staten Island parents were also assigned to each of four other rearing conditions: a) paired with an individual from the same source population; b) paired with an individual from the other source population; c) in a group box with three others from the same source population; or d) in a group box with one individual from the same source population and two from the other source population. All of the individuals derived from the Staten Island population were spotted whereas all of the Tomales Bay descendants were immaculate. Redwood bark mulch was added to each box to encourage egg-laying. Slugs were weighed biweekly and cleaned and fed weekly (as in Leonard *et al.* 2002), so that all boxes were checked for eggs at least weekly. Clutches found were treated as described above. Hatchlings were weighed when found and preserved in 95% ethanol for future genetic analysis.

Behavioral observations

All observations on sexual behavior reported here for *Ariolimax buttoni* (see Table 3) resulted from casual observations of UCSC animals held in group boxes. Where animals were observed to be copulating when the box was opened for cleaning, notes were made and/or the behavior was videotaped as time allowed. Where possible, observations were continued for 30 min after the end of the copulation. The copulation of Mendocino individuals on April 10, 2001 was noted but not followed for any length of time.

RESULTS

Phally status

Mills College animals

Only 7 of the 67 Mills College slugs (4 of the 55 slugs collected on the Mills College campus, and 3 of the 12 slugs collected at Leona Park), were found to have a penis and, of those, one only had a penis fragment (Table 1). In 59 of the 60 individuals lacking a penis, the vas deferens ended abruptly in the atrial mesentery (Fig. 1). In 20 of these 59 individuals, there was a small bulb at the end of the vas deferens as described by Pilsbry and Vanatta (1896). The apical termination of the vas deferens in the individual with a fragment of a penis was on the penis fragment. This specimen was in the immature reproductive state (see below). None of the 17 Mills College juveniles hatched in the laboratory had a penis.

UCSC animals

A total of 16 individuals were dissected (Table 2). All of the individuals derived from Tomales Bay populations and the individuals collected from UCSF and Central Marin had a fully developed penis with an apical retractor muscle and a long vas deferens that terminated in the penis (Fig. 2B). None of the 10 individuals derived from the Staten Island population had a penis (Fig. 2A), including two individuals that spent their entire lives in isolation. Of these 10 individuals, 5 had a small stub where the penis would be in a euphallate individual (Fig. 2A) whereas the remaining 5 lacked any trace of a penis (Fig. 1). All 10 individuals lacked a penial retractor muscle and 9/10 had a blind termination of the vas deferens. In one individual the vas deferens ended in a bulb as was seen in many of the Mills College animals (Table 1). The vas deferens connected to the base of the penial stub in one individual.

Reproductive development

Mills College animals

Measurements and appearance of the individual organs of the reproductive tract for the 67 dissected adult Mills College slugs are given in Westfall (1952). On the basis of the color and appearance of the organs and the length of the albumen gland, four reproductive states were identified: 1) immature, 2) intermediate (or "sperm producing"), 3) hypertrophied (or "egg-laying"), and 4) "old" (Appendix). The reproductive state in the dissected slugs varied with season (Fig. 3). In the spring most (10/12) individuals were in the intermediate state (Fig. 3) with an albumen gland of small to intermediate size. In contrast, in the fall and winter there was a clear division into two groups of individuals: those in the immature state with a very small albumen gland and those in the hypertrophied state with a very large albumen gland. Slugs collected in October and kept in captivity were also found to be more often in the hypertrophied state as the date of dissection moved from November to February.

There was no relationship between season or reproductive state and the presence of a penis. Four of the individuals with a penis were in the immature reproductive state, one in the intermediate state, and two others in the hypertrophied state (Appendix; Fig. 3). In addition, there was no relationship between body weight and the presence of a penis; the second smallest and third largest animals dissected had a penis (Fig. 4). Each reproductive state included individuals with a broad range of weights although the smallest animals were in the immature state and the largest in the hypertrophied state (Fig. 4).

The reproductive tract was not distinguishable in juveniles for two weeks after hatching. After two weeks, the organs of the reproductive system were in the immature state with a hair-like hermaphroditic duct, leading from a small, white, smooth, lobed ovotestis and a small albumen gland. After a short distance this duct divided into a free oviduct and a vas deferens. These ducts were straight and hair-like in younger specimens but began to show more coiling in older specimens. In older juveniles the vagina and vas deferens terminated jointly in the atrium (as in Fig. 1) but there was no indication of a penis even at 19 weeks of age when other reproductive organs were well developed. Juvenile slugs of 15-19 weeks of age weighed between 3 and

Table 1. Summary of morphological data on adult *Ariolimax buttoni* from Mills College in Oakland, California

Phally status	Number of slugs	Weight (mean ± SD)	Number in immature state*	Number in intermediate state*	Number in hypertrophied state*	Number with state unclear
Penis present	6	32.12 ± 129 g	3	0	2	1 (state questionable)
Penis fragment	1	37.5 g	1	0	0	
No penis but bulb on end of vas deferens	20	32.4 ± 9.08 g	2	9	8	1 (state questionable)
No penis and no bulb	39	29.3 ± 5.62 g	18	13	8	
Unknown	1	24.5 g				(insufficient information)
Weight (mean ± SD)			28.42 ± 6.61 g	30.49 ± 6.27 g	33.31 ± 9.52 g	32.53 ± 9.91 g
Total	67		24	22	18	3

* See Appendix for definition of reproductive states.

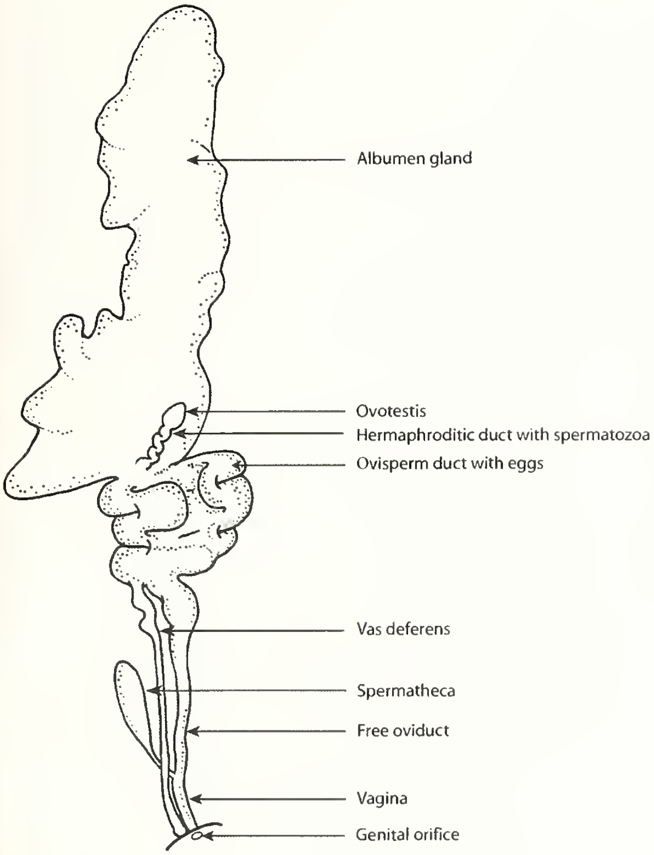

Figure 1. Drawing of a dissected aphallate individual of *Ariolimax buttoni* which had been observed to lay eggs before dissection; hypertrophied reproductive state (see Appendix) with sperm present in the hermaphroditic duct and the oviduct distended with eggs. The hermaphroditic duct proceeds from the ovotestis (=hermaphroditic gland = gonad) carrying both eggs and sperm. After the albumen gland, the term ovisperm duct is used for a convoluted conjoined vas deferens and oviduct. After the vas deferens branches off (to the penis in euphallate specimens, see Figure 2B) to end near the atrium here, the ovisperm duct continues as the free oviduct to the point of attachment of the spermatheca (=bursa copulatrix = gametolytic gland) with the vagina. The free oviduct is usually a wide, convoluted duct while the vagina is a short, straight tube from the spermatheca to the atrium, and is divided into two portions by a small, thick, annular muscle (intrinsic muscle of the vagina).

6.3 g (Westfall 1960). The juvenile slugs all showed the immature reproductive state (Appendix), except that the very first juvenile examined, dissected at four weeks of age (representative weight, 0.7 g, Westfall 1960), was found to have a relatively large, loosely lobed, ovotestis. The reason for the different appearance of this one individual is not known.

UCSC animals

The spermatheca was reddish in most individuals from Staten Island. All Tomales Bay animals had a small spermatheca and in 3 of the 4, it was pale in color. While all of the isolated, dissected animals from Staten Island (3/8), laid one or more clutches of eggs, none of the isolated Tomales Bay animals laid eggs (see below).

Egg-laying

Results on egg-laying and hatching in the Mills College animals have been published elsewhere (Westfall 1960). In UCSC animals, egg-laying by animals hatched in the fall-winter of 2003-2004 from Staten Island and Tomales Bay parents, began in October 2004 and continued until late February 2005. Egg-laying then paused for this group of slugs until mid-August 2005 when it resumed and continued until late February 2006. However, only in one box of four individuals did egg production occur in both seasons (2004-2005 and 2005-2006).

Three individuals from Staten Island parents, isolated since hatching, laid eggs (only two were dissected and listed in Table 2). One individual, from a clutch of 13 November 2003, produced three clutches for a total of 89 eggs: 32 eggs on 13 December 2004 (Clutch 1A), 13 eggs on 20 December 2004 (Clutch 1B), and 45 eggs on 9 February 2005 (Clutch 1C). This individual was found dead on 4 August 2005. All three clutches developed normally and produced a total of 45 viable hatchlings. Clutch 1A produced 21 hatchlings between 31 January and 7 February 2005. Clutch 1B produced 6 hatchlings from three eggs between 3 and 9 February 2005: one egg produced a single hatchling, one produced twins, and one produced triplets. The production of more than one hatchling from an egg has also been seen in other species of *Ariolimax* (Leonard *et al.*, unpubl. data; B. Miller, pers. comm.). Clutch 1C produced 18 hatchlings 24-28 March 2005.

The second individual (from a clutch found 28 November 2003) laid a total of 120 eggs, 101 which hatched: a) 28 eggs found 12 October 2005, 16 of which hatched; b) 17 eggs found 18 November 2005, 18 of which hatched, including one set of twins; c) 56 eggs found 8 December 2005, 50 of which hatched; and d) 19 eggs found 17 February 2006, 17 of which hatched. The third individual, from a clutch of 13 November 2003 [a clutch mate of the first individual (above)], was found with a clutch of 80 eggs on 8 December 2005, which produced 66 hatchlings, and a clutch of 56 eggs on 19 January 2006 which produced 55 hatchlings. The latter two individuals survived until frozen on 22 June 2006, and were found to be aphallate when dissected (Table 2).

A box of four individuals, two from Staten Island parents and two from Tomales Bay parents produced a clutch of 17 eggs on 14 October 2004. The oldest individual in the box

Table 2. Summary of dissections of *Ariolimax buttoni* held at UCSC at Santa Cruz, California

Population	Isolate/Group-held/Collected?	Penis	Vas deferens	Spermatheca	Egg laying
Central Marin	Collected as adult	Very large bulbous	Connects to penis		Never laid eggs in lab
UCSF	Collected as adult	Very thick	Connects to penis	Sac-like	Never laid eggs in lab
Staten Island	Collected as adult	No trace of penis	Blind termination	Reddish, flaccid	Never laid eggs in lab
Staten Island	Reared in isolation throughout life	Small penial stub	Blind termination	Reddish	Laid fertile eggs
Staten Island	Reared in isolation throughout life	Small penial stub	Blind termination	Reddish, sac-like	Laid fertile eggs
Staten Island	Laboratory reared, paired at 6 months of age	Splayed penial stub	Blind termination	Dark red	3 clutches of fertile eggs from pair
Staten Island	Laboratory reared, paired at 6 months of age	No trace of penis	Ends in genital pore		3 clutches of fertile eggs from pair
Staten Island	Laboratory reared, paired at 6 months of age	No trace of penis	Connects to base of penial stub near atrium	Red, flaccid	Fertile eggs laid by pair
Staten Island	Laboratory reared, grouped at 6 months of age	No trace of penis; 1 cm of vagina everted	Blind termination	Large	Several clutches of fertile eggs laid by group
Staten Island	Laboratory reared, grouped at 6 months of age	Small penial bulb	Blind termination	Dark reddish	Several clutches of fertile eggs laid by group
Staten Island	Laboratory reared, grouped at 6 months of age	No trace of penis	Ends in bulb		Several clutches of fertile eggs laid by group
Staten Island	Laboratory reared, grouped at 6 months of age	No trace of penis; 0.5 cm of genital pore everted	Blind termination	Large, flaccid, red	Several clutches of fertile eggs laid by group
Tomales Bay	Reared in isolation throughout life	Penis present	Connects to penis	Flabby, small	Never laid eggs
Tomales Bay	Reared in isolation throughout life	Penis present	Connects to penis	Reddish, moderately small	Never laid eggs
Tomales Bay	Reared in isolation throughout life	Penis present	Connects to penis	Small, pale	Never laid eggs
Tomales Bay	Laboratory reared, paired at 6 months of age	Penis present	Connects to penis	Small, creamy, firm	Never laid eggs

came from a clutch of 13 November 2004, so the parent was no more than 11 months old at the time of egg-laying. This clutch produced 3 hatchlings. On 11 November 2004, a clutch of 29 eggs was found in a box containing a pair of individuals, both from Tomales Bay parents, one from a clutch found 30 December 2003 and the other from a clutch found 2 January 2004. This means that the individual laying the eggs could not have been older than 10.5 months from the date the egg was laid. Fourteen of the 29 eggs in this clutch hatched. A second clutch of 12 eggs found in this box on 13 December 2004 produced 6 hatchlings. On 24 November 2004 the Staten Island (spotted) individual of a pair of one Staten Island (spotted) and one Tomales Bay (immaculate) slug, was found laying eggs. A total of 54 eggs were found in the box at that time, although it is not clear that they were all laid by the spotted mother. Forty-one hatchlings were produced from this clutch. The spotted slug was hatched from a clutch found 16 January 2004 and so was approx. 10 months and eight days in age (from the egg). Nine more eggs were found in this box on 24 November 2004 and this clutch produced 2 hatchlings.

Sexual Behavior

Little is known about sexual behavior in *Ariolimax buttoni*. To date, we have never observed a complete sexual interaction from courtship to the termination of copulation

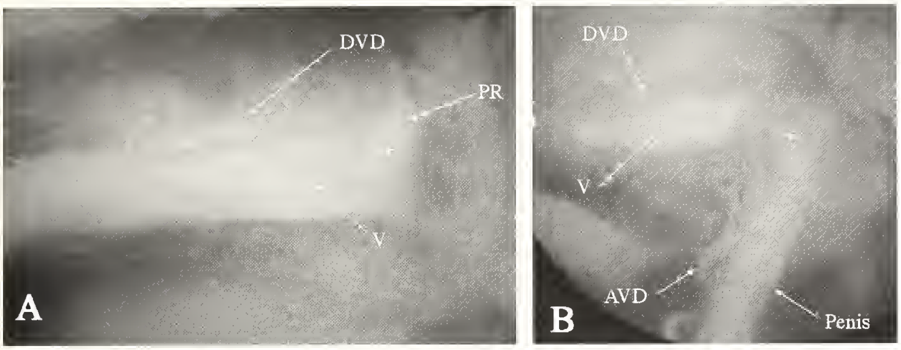

Figure 2. Photographs of dissections of aphallate (A) and euphallate (B) individuals of *Ariolimax buttoni.* Individual A was laboratory reared from Staten Island parents. Individual B was laboratory reared from Tomales Bay parents. AVD, ascending vas deferens; DVD, descending vas deferens; P, penis; PR, penis stub; V, vagina; *, location of atrium.

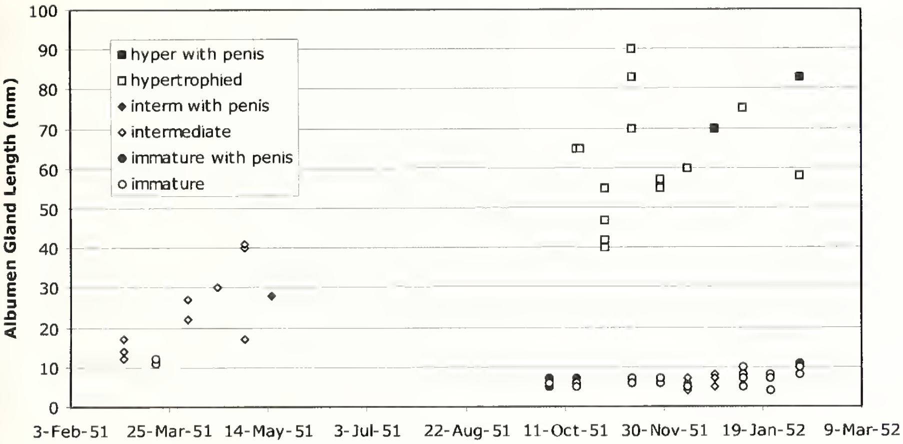

Figure 3. Relationship between reproductive state (Appendix), season, and albumen gland length in 66 individuals *Ariolimax buttoni.*

in this species. However, UCSC animals maintained in the laboratory in groups have occasionally been found *in copula. Ariolimax buttoni*, like *A. columbianus*, and unlike other species of *Ariolimax*, has both spotted (maculate) and unspotted (immaculate) individuals, and three of the copulating pairs observed in this study involved both a maculate and an immaculate individual (Fig. 5; Table 3). Copulations between maculate and immaculate individuals are also seen in *A. columbianus* (Cody 2006).

In the laboratory, copulations have been observed from February through early September. Copulation was observed on 11 or 12 (see below) occasions involving animals from 5 different populations (Table 3). In one case, a pair of slugs from Central Marin was found copulating unilaterally at 11:07 pm on 5 September 2002 (by JSP), observed until 12:30 am 6 September 2002, and then left alone overnight. What was apparently the same pair of slugs, in the same position, was found copulating when the box was next checked at 2:30 pm on 6 September 2002 and the pair finally separated at 10 pm that evening. We cannot say with confidence whether this represents one or two copulations. If we count it as two copulations, of the 12 copulations that have been observed, 7 involved simultaneously reciprocal copulation between the members of a pair whereas 5 were unilateral. If the interactions of 5-6 September 2002 are counted as one copulation, then 7/11 copulations observed have been simultaneously reciprocal. In three cases, the termination of the copulation was observed and in all of these cases, the copulation was unilateral when first observed. Of these, one terminated 62 min after the copulation was noticed, one terminated 98 min after first observed and one (5-6 September 2002) terminated 7.5 h after observation resumed on the second day; 22 h and 53 min after the pair was first seen copulating. Reciprocal copulations may become unilateral when one penis is withdrawn considerably earlier than the other (Leonard *et al.* 2002). The available data (Table 3) show that copulations in *Ariolimax buttoni* are often very long. In 3/12 observations copulation lasted more than 7 h, and in one case (5-6 September 2002), the interaction may have lasted almost 23 h. In the unilateral copulation on 5 February 2001, between two immaculate individuals collected from the UCSF campus, when the pair were first seen, one slug had intromission as a male and the second slug (acting as female) had its penis completely everted and resting against the body of its partner. The end of the penis was greatly enlarged in the form of a broad bulb. As the second slug withdrew its penis, the bulb gradually deflated and the penis involuted from the tip. In the observations of 5-6

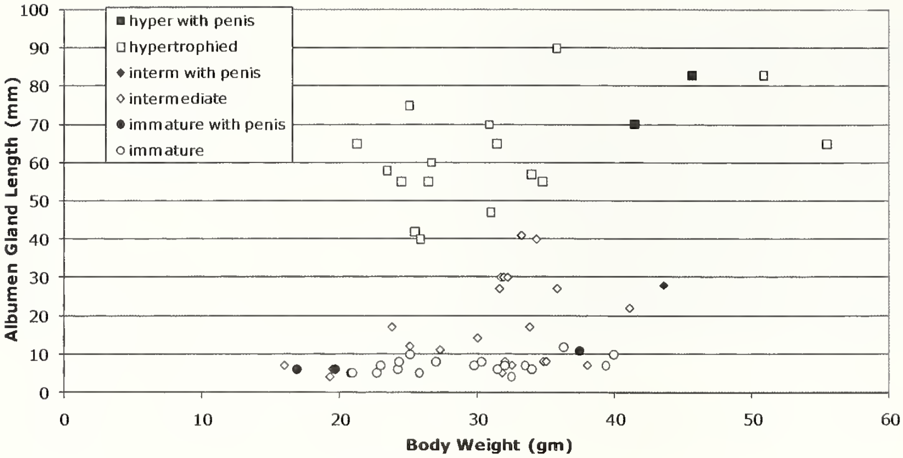

Figure 4. Relationship between reproductive state (Appendix), body weight, and albumen gland length in 66 individuals of *Ariolimax buttoni.*

Table 3. Copulations of *Ariolimax buttoni* from different populations in California.

Population	Date	Time first observed	Observations ended	Type of intromission
UCSF	2/5/2001	16:05	17:43	Unilateral
Mendocino	4/10/2001	17:45	17:46	Simultaneously reciprocal
Mendocino	5/3/2001	17:30	20:29	Simultaneously reciprocal
Mendocino	5/17/2001	21:05	22:23	Simultaneously reciprocal
Central Marin	7/17/2002	13:15	15:17	Simultaneously reciprocal
Central Marin	9/5/2002	23:07	24:30	Unilateral, spotted male
Central Marin	9/6/2002	14:30	22:00	Unilateral, spotted male
UCSF	4/8/2003	18:31	26:01	Simultaneously reciprocal
UCSF	5/5/2003	11:52	23:35	Simultaneously reciprocal
Tomales Bay	5/6/2003	16:04	18:28	Unilateral
Tomales Bay	6/19/2003	15:45	16:47	Unilateral
Tomales Bay	8/8/2003	13:44	18:30	Simultaneously reciprocal

September 2002 one slug was seen to contact the penis with its mouth on several occasions but there was no evidence of chewing on the penis.

DISCUSSION

Phally Polymorphism

Since Heath's (1916) description of apophallation in *Ariolimax californicus*, the tendency has been to explain absence of a penis in this genus in this way (Mead 1942, 1943). However, Heath himself expressed doubt of this interpretation (cited in Mead 1943, see above). The current study was stimulated by the observation that all 27 individuals of *Ariolimax* collected on the Mills College campus for an anatomical study lacked a penis (Paull 1951). This is a higher incidence of aphally than would be expected from the 5% apophally rate reported by Heath (1916) for *A. californicus.* Only 7 of 67 slugs collected from Mills College in the current study had a penis. In all cases the aphallate slugs were lacking not only a penis but all penial musculature, and the vas deferens was connected to tissue at or near the atrium (Fig. 1), terminating in a bulb in many cases, which is not what one would expect from apophallation (see discussion by Roth 2004). Further evidence that aphally in this population is not derived from apophallation during copulation comes from the observation that none of a series of sexually immature laboratory-reared slugs from Mills College, which were up to 4 months old, showed any signs of development of a penis. This led to the hypothesis that aphally occurred naturally in some individuals of *A. buttoni,* and that an innate phally polymorphism rather than apophallation was largely responsible for the presence or absence of a penis in this and perhaps other species of *Ariolimax.*

This hypothesis was confirmed by the observation that in individuals derived from the Staten Island population, aphally was found even in two individuals that were reared in isolation from the egg to the age of 30 months and had both laid eggs. Aphally in these individuals could not be due to either apophallation or sexual immaturity. Moreover, the anatomy of these aphallic isolates was consistent both with that of other Staten Island individuals that were group-housed as adults and with that of the Mills College animals. The aphallates are characterized by either the complete absence of a penis (Fig. 1) or reduction of the penis to a small stub (Fig. 2A); in both cases, the penial retractor muscle is absent and the vas deferens ends blindly in a mesentery or in the neighborhood of the atrium. The description of dissection of a freshly apophallate *A. dolichophallus* by Mead (1942) suggests, however, that the distinctions between aphally as described in Fig. 1 and 2, and a healed apophallate individual, are subtle.

The anatomy of *Ariolimax buttoni*, as described by Pilsbry and Vanatta (1896), and the aphallate individuals from Mills College described here (Fig. 1, also Paull 1951) and the Staten Island animals (Fig. 2A), is very consistent with the descriptions and illustrations of aphallic individuals in other stylommatophorans (Watson 1934, Riedel 1955, Tompa 1984, Pokryszko 1987, see discussion in Roth 2004). Phally polymorphism, in which a hermaphroditic population or species consists of a mixture of individuals with normal penes (euphallic individuals) and individuals with either markedly reduced penes (hemiphallic individuals) or no penis at all (aphallic individuals) is widespread in the Pulmonata and has evolved many times (see discussion in Duncan 1975, Tompa 1984, Pokryszko 1990, Lace 1992, Schrag and Read 1992, Viard *et al.* 1997, Doums *et al.* 1998, Backeljau *et al.* 2001). The results presented in this study offer a clear demonstration of phally polymorphism in *A. buttoni*.

Aphally seems to be common in at least some populations of *Ariolimax buttoni.* Pilsbry and Vanatta (1896, discussion in Pilsbry 1948) examined a large collection of individuals lacking a penis from Oakland, and Heath (Mead 1942, cited above) dissected 400 aphallate specimens from Hog Island in Tomales Bay. Westfall (1952) also reported that of 85 specimens collected at Mills College in the spring of 1952 (subsequent to the work described here), only 9 had a penis, of which 3 were in the hypertrophied state and 6 in the immature reproductive state, in the terminology used here. It is characteristic of phally polymorphisms that the percentage of a given morph varies widely from one area to another and from season to season (Lace 1992). Baur and Chen (1993) found that the frequency of aphallic individuals in populations of *Chrondrina avenacea* (Bruguière) varied from 0.9% to 89.2% in the vicinity of Basel, Switzerland. The tendency for the frequency of phally morphs to vary with environmental conditions (*e.g.*, Watson 1934, Schrag and Read 1992, Baur *et al.* 1993) may explain why Mead (1942, 1943) found only euphallate individuals in the vicinity of Hog Island whereas Heath had found only aphallate individuals at Hog Island earlier. In this study, all four individuals derived from populations near Tomales Bay were euphallate (Table 2).

The results presented here, then, demonstrate that phally polymorphism is present in *Ariolimax buttoni*, showing that only 7/67 Mills College and 4/16 UCSC, including 0/10 Staten Island, slugs had a penis, and leave open the question of whether apophallation occurs in this species. Aphallate individuals have also been found in a population of *Ariolimax (Meadarion) brachyphallus* from Hillsborough, San Mateo City, CA (Pearse, *unpubl. data*). Wright (1938) stated that 415 individuals of *Ariolimax californicus* he examined did not have a penis, or other "male parts" whereas 248 individuals were found to have normal, "or regenerating" penes. Wright also stated that the aphallic individuals were the result of apophallation but provided no evidence to support this, nor did he provide details of the anatomy or even information as to the source of these specimens. Wright's observation suggests that an anatomical study of that species is needed to determine whether aphally as well as apophallation occurs. It is possible that phally polymorphism will be found to be more widespread in *Ariolimax.*

The adaptive significance of phally polymorphism is not entirely clear. Local Mate Competition theory (Charnov 1982) predicts that allocation to male function in hermaphrodites should be reduced where few sexual partners are available, suggesting that aphally should be more common where self-fertilization is common or population densities are low. Baur *et al.* (1993) hypothesized that aphally evolves in populations that typically self-fertilize. In both cases, aphally is predicted to be associated with a capacity for uniparental reproduction and the role of environmental factors in influencing the ratio of aphallic to euphallic offspring is hypothesized to be an adaptation to colonizing new habitats (Schrag and Read 1992, but see Baur *et al.* 1993)

Uniparental Reproduction

Uniparental reproduction, either by self-fertilization, as has been widely assumed, and well-documented in some cases, or by apomixis, as suggested by some authors (McCracken and Selander 1980, Foltz *et al.* 1982a, Hoffmann 1983) is widespread, although not universal in stylommatophorans (Tompa 1984, Heller 2001). Within a genus, some species may be obligate outcrossers whereas others readily self-fertilize (Foltz *et al.* 1982b, Reise 2002). Mead (1942) reared *Ariolimax* in isolation for as long as two years without the production of any eggs but considered the question of uniparental reproduction in *Ariolimax* still open. Here we report that *A. buttoni* can produce viable offspring with high rates of hatching success without cross-fertilization. Uniparental reproduction has also been observed in *A. dolichophallus* but with low hatching success (Miller and Sinervo 2007). Westfall (1952) reported that in Mills College animals, the development of the ovotestis did not differ between euphallic and aphallic individuals; that is the qualitative degree of sperm vs. egg production did not appear to depend on phally status. The production of sperm observed in aphallate individuals in histological studies (Westfall, 1952), suggests that self-fertilization could occur in *A. buttoni.* Similar results have been reported in the genus *Zonitoides* Lehman, 1862 (Watson 1934). Resolution of the question of whether these offspring are the result of selfing or apomixis will require genetic analysis.

Sexual Behavior

Of the observations reported in Table 3, about half of the pairs had reciprocal intromissions and the other half had unilateral intromissions, in which one slug was acting as a female and the other as a male. Copulation lasted more than 7 h after being first observed in 3 of 12 observations, and in one case (5-6 September 2002) the interaction may have lasted almost 23 h after detection (see above; Table 3). Thus, copulation in *Ariolimax buttoni* often lasts more than the two hours typical of simultaneously reciprocal intromissions of *A. dolichophallus* and much longer than the brief, unilateral intromissions of *A. californicus* (Leonard *et al.* 2002). In long reciprocal copulations such as those of *A. dolichophallus* (Leonard *et al.* 2002) it is not unusual for one individual to withdraw the penis long before the other (Leonard, unpublished observation). Therefore, it seems likely that copulations between euphallate *A. buttoni* (Figs. 5A-C) are normally long and simultaneously reciprocal. The unilateral intromissions observed here may have been the end of simultaneously reciprocal copulations or they may have involved copulation with a partner that lacked a penis (below).

Life History

The life histories of *Ariolimax* spp. are poorly known. The observations reported here provide the most detailed picture available on sexual development and the reproductive cycle for any species of *Ariolimax.* We found that, in the laboratory, *Ariolimax buttoni* may live more than 30 months. This is consistent with previous reports that individuals of *A. californicus* and *A. dolichophallus,* collected as adults, survived more than 18 months in the laboratory (Leonard *et al.* 2002). The anatomical and histological data obtained in the Mills College study indicate that, in Oakland populations, individuals may live more than a year in the field, since two classes of individual were found in fall and winter dissections; spring and summer specimens showed a gradual increase in albumen gland length, suggesting the maturation of a single cohort (Fig. 3). This fits well with field data (Pearson *et al.* 2006) for identifiable individuals in a population of *A. buttoni* in Orinda, Contra Costa Cty, CA that showed a life span of approx. 2 years.

The data also provide a clear picture of the phenology of *Ariolimax buttoni.* Both UCSC (Leonard *et al.* unpublished) and Mills College animals held in the laboratory (Westfall 1952, 1960) laid eggs in the fall and winter as suggested for *Ariolimax* by Mead (1942). Mills College animals with reddish-brown ovotestes, a spermatheca filled with reddish fluid, and a yellow albumen gland larger than the ovotestis (the hypertrophied reproductive state), believed to represent the egg-laying stage, were first seen in a dissection of 22 October 1951 and then seen regularly until February 1952 (Fig. 3). Egg-laying and juvenile growth from individuals in the Mills College portion of the study have been reported elsewhere (Westfall 1960). Westfall (1960) reported a minimum time to hatching of 23 days and a maximum of 2 months for Mills College slugs, which brackets the 47-54 days found for UCSC animals (Leonard *et al.* unpublished). In *A. dolichophallus,* hatching time ranged from 51-55 days and in *A. californicus* from 46-81 days (Leonard *et al.* 2002). These hatching times all reflect laboratory conditions and will probably depend strongly on temperature, making it likely that hatching times in the field will be somewhat longer. In the spring and summer, animals were found to be in the immature and intermediate reproductive states, whereas in fall and winter all three states were found (Fig. 3). This is consistent with data from Paull (1951) who noticed that the reproductive system was more often in the immature state from February to April, whereas hypertrophy of the reproductive tract was predominant in *A. buttoni* collected from September to the middle of February. In field observations, juveniles of *A. buttoni* weighing less than 1 gram appeared in January (Pearson *et al.* 2006).

Since laboratory data demonstrate that *Ariolimax buttoni* can live more than one year, with some individuals laying eggs at less than one year of age while some clutch mates reared under the same conditions do not lay eggs until over two years of age (see below), it seems likely that not all individuals will reproduce in their first year, perhaps accounting for the occurrence of individuals in the immature and intermediate reproductive states throughout the year in this study. Protandrous development of the ovotestis is typical of stylommatophorans (Tompa 1984) and has been reported for *A. californicus* (Gottfried and Dorfman 1970), making it seem probable that development in *A. buttoni* may involve a progression from the immature reproductive state through the intermediate male state to the hypertrophied female state, and then the old reproductive state, although the intermediate state may be heterogeneous. The intermediate reproductive state is the state characterized by massive sperm production (Westfall 1952; Appendix) and one would expect it to be associated with copulation. Our observations suggest that in *A. buttoni* may become sexually mature and begin copulation as early as the summer of their first year and lay eggs that same fall. The first specimen found to have an albumen gland greater than 20 mm in length in the Mills College study was dissected in April 1951 (Fig. 3). The first

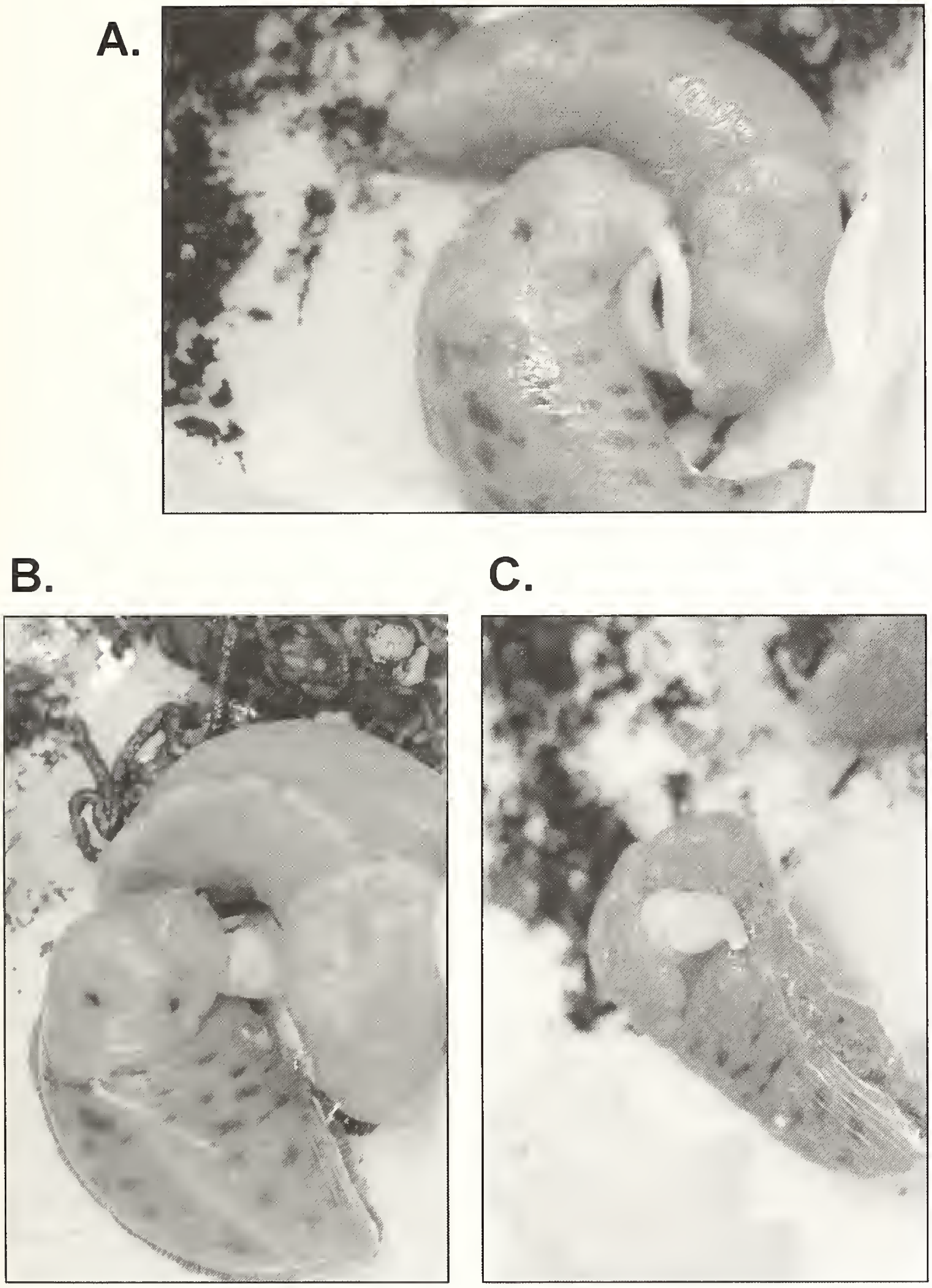

Figure 5. A. A copulating pair of euphallate individuals of *Ariolimax buttoni*. Both individuals were collected from the UCSF campus and held in a group box; found copulating when the box was opened for cleaning. B. The same pair of individuals later in same copulation, with at least one penis inflated. C. One individual of same copulation with short, rigid withdrawn penis still everted after withdrawal from partner.

individual found to have a reddish and enlarged spermatheca (= bursa copulatrix = gametolytic gland) was dissected 17 October 1951. The spermatheca, often termed the gametolytic gland (Tompa 1984, Gómez 2001), serves to digest both allo- and autosperm in stylommatophorans, as well as stray oocytes and excess secretions. The reddish color of the spermatheca may, therefore, indicate copulation and/or egg-laying. Individuals from the Staten Island population that were reared in isolation but had laid eggs had a reddish spermatheca (Table 2) but one of the individuals from Tomales Bay, also reared in isolation but having never laid eggs, also had a reddish spermatheca. Laboratory observations from UCSC animals (Table 3) show copulations occurring between February and September. This is consistent with field observations from *A. dolichophallus* in which observations of copulating pairs ranged from February to mid-October (Leonard *et al.* 2002).

Laboratory data from the UCSC animals indicate that the rate of maturation, at least in terms of egg-laying, varies widely. The age at first egg-laying has varied from slightly over 10 months to more than 24 months among individuals that have laid at least one clutch and many animals in the study had not yet laid eggs at 30 months of age (Leonard *et al.* unpublished), as Mead (1942) also reported. Gottfried and Dorfman (1970), reported that individuals from a population of *Ariolimax californicus* from Portola Valley, San Mateo Co., California, reared in the laboratory, showed protandrous gonadal maturation with individuals having immature gonads at body weights of less than 10 g and individuals of 20-30 g and approx. 12 months of age having "maturing male phase" gonads. The first "intersex" gonads appeared at a body weight of 40-45 g and an approx. age of 24 months and fully female gonads were associated with a body weight of 55-60 g in weight and 36 months of age. These data are similar to those from individuals in the current study (Staten Island isolates, Table 2) that first laid eggs at two years of age, although there was in general no clear relationship between weight and reproductive state in the Mills College animals (Table 1; Figure 4). In a study of identifiable individuals in the field, Pearson *et al.* (2006) found a correlation between age and weight only up to 20 g for *A. buttoni*. Gottfried and Dorfman's (1970) data differ substantially from the results obtained for *A. californicus* by Leonard *et al.* (2002) using the same rearing conditions as used for the UCSC animals in the current study, where lab-hatched individuals were found to begin copulating at 8 months of age, laying eggs as early as 50 weeks of age, and achieving a much higher growth rate than reported by Gottfried and Dorfman (1970). Lab-reared *A. californicus* were observed to copulate as early as eight months of age (from the egg) and to lay eggs at 12 months (Leonard *et al.* 2002) and *A. (Ariolimax) stramineus* Hemphill, 1891 and *A. (Meadarion) brachyphallus* Mead, 1943 have also been observed to lay eggs at less than 12 mo of age in the laboratory (Leonard *et al.* unpublished). In contrast, Mead (1942, p. 116) reported that the genital system was still "very minute and completely non-functional" in *A. brachyphallus* at the end of one year and that sexual maturation (ability to copulate as a male and to receive sperm but not lay eggs) was reached in *A. dolichophallus* at approx. 18 mo. Miller and Sinervo (2007) reported large variance in growth of *A. dolichophallus* in the laboratory, as seen by Leonard *et al.* (2002) for *A. californicus*. This may be associated with variance in sexual development as seen here in *A. buttoni*.

Reproduction in stylommatophorans appears to be strongly influenced by environmental factors (Potts 1975, Tompa 1984, South 1992, Gomot de Vaufleury 2001). Moreover, growth rates may vary greatly among clutch mates reared under the same conditions (Shibata and Rollo 1988, Leonard *et al.* 2002, Miller and Sinervo 2007), and, as seen in the current study, the age at first egg-laying may vary by more than 12 months among clutch mates. Consequently, variation from year to year and site to site would not be unexpected in *Ariolimax buttoni*. However, in general the life cycle seems to involve egg-laying in fall and winter with hatching in late winter to early spring, and some individuals copulating as early as the late summer or fall of their first year and laying eggs in the late fall or winter. Overwintering individuals may copulate in the spring or summer of the second year and lay eggs that fall or winter. Whether regression of gonads occurs in *A. buttoni* (see discussion in Mead 1942, Westfall 1952) is not clear. Also, we do not know whether an individual will lay eggs in successive years. Some stylommatophorans have an iteroparous life cycle, with cycles of gonadal development and reproduction in successive years whereas other species have a pattern whereby individuals have a more or less prolonged semelparous life cycle (perhaps over several years) and gonadal development is not reversible once sexually mature (see discussion in Heller 2001). The available data do not allow us to distinguish between these models for *A. buttoni*, or other species of *Ariolimax*.

Sexual Selection and the Sexual Biology of *Ariolimax buttoni*

In order to understand the potential for sexual selection, it is important to understand the age at first reproduction, the duration of the reproductive life span, the type of mating behavior, and the potential for uniparental reproduction. The potential strength of sexual selection is measured by the variance in reproductive success among individuals (see review in Leonard 2006), and one factor that would tend to increase the potential for sexual selection in a species is a skewed sex ratio. While in simultaneous hermaphrodites the operational sex ratio, or sex ratio at the time of mating, is considered to be 1:1, if there were a delay between mating and egg-laying there may be a skew in breeding sex ratio (Arnold and Duvall 1994). In *Ariolimax*, *A. californicus* have been observed to copulate as young as 8 months of age and to begin egg laying at 11.5 months of age (Leonard *et al.* 2002). This delay suggests that not all individuals that copulate will lay eggs, creating a potential skew between the number of individuals in the population that sire young and those that are mothers of young, which would tend to increase the variance in reproductive success among individuals and the potential for sexual selection. The range in age at first egg-laying seen in *A. buttoni* in the current study (from 10 to more than 24 months) further suggests that variance in female reproductive success will be substantial. Phally polymorphism may also contribute to skewed breeding or even operational sex ratios in that aphallates will be unable to copulate as males with other individuals although they may be able to copulate as females (but see Reise 2007). The anatomy of aphallic individuals in *A. buttoni*, with the vas deferens ending blindly or in a bulb seems to be inconsistent with transfer of sperm to conspecifics. However, observations of copulation in the female role by apophallate *A. dolichophallus* (Leonard *et al.* 2002) suggest that aphallate individuals may be able to copulate as females, receiving sperm from euphallate partners. In the stylommatophoran, *Vertigo pusilla* O.F. Müller, 1774, Pokryzsko (1990) found that aphallates copulated as females with euphallate individuals. In the basommatophoran *Bulinus truncatus,* in which self-fertilization is the predominant mode of reproduction, phally status (euphallic or aphallic) was not correlated with offspring heterozygosity, indicating that aphallic individuals are as likely to have their eggs fertilized by a conspecific as were euphallic individuals (Doums *et al.* 1996, Viard *et al.* 1997). In a mixed population of aphallates and euphallates with outcrossing, the euphallates should have greater potential reproductive success in the male role, increasing variance in reproductive success and the potential for sexual selection. However, if reproduction in *A. buttoni* is predominantly uniparental, the opportunity for sexual selection would be limited.

Sexual behavior also offers indications of the strength of sexual selection. The very long (>7 h) copulations observed in this study (Table 3) suggest that sexual behavior in *Ariolimax buttoni* may involve higher expense and/or risk than in species with shorter copulations. Sexual selection in hermaphrodites has been hypothesized to involve resolution through reciprocity between members of a pair of a sexual conflict over sexual role (Axelrod and Hamilton 1981, Leonard 1990, recent reviews by Michiels 1998, Leonard 2005, 2006). In *A. dolichophallus* the mating system is based on simultaneously reciprocal copulation between the members of a pair (Leonard *et al.* 2002). In *A. californicus* intromissions are unilateral but occur in bouts and the members of a pair are hypothesized to alternate roles during these bouts, creating reciprocity (Leonard *et al.* 2002). In the data presented here, about half of the observed copulations were simultaneously reciprocal (Fig. 5A) and since in other species of *Ariolimax* simultaneously reciprocal copulations often involve a period of unilateral intromission after one individual withdraws (Leonard *et al.* 2002, Leonard and Pearse, *unpubl. obs.*), we hypothesize that simultaneously reciprocal copulations will be found to be the rule in *A. buttoni* where both individuals are euphallate.

If reciprocity were important to the mating system, as predicted by the Hermaphrodite's Dilemma model (Leonard 1990), then individuals able to mate in only one sexual role, as is assumed to be the case for aphallates (but see Westfall 1952), should be less desirable as mating partners than euphallates. If on the other hand, the reciprocity seen between euphallates is "unconditional" and an artifact of both hermaphrodites being eager to act as males as predicted by Michiels (1998, see discussion in Leonard 2005), then aphallates should be as desirable as euphallates as sexual partners. *Ariolimax buttoni,* offers an exciting opportunity to distinguish between these models. Additionally, the capacity for uniparental reproduction should allow hermaphrodites to avoid Game of Chicken conditions in the Hermaphrodite's Dilemma (Leonard 1990). Therefore, in a species with the option of uniparental reproduction, the Hermaphrodite's Dilemma predicts that reciprocity will be conditional on the partner's behavior (see Leonard 2005). The evidence for phally polymorphism and uniparental reproduction in *A. buttoni* creates an interesting opportunity to test the predictions of theory. Genetic studies will be required to distinguish between hypotheses and to measure the variance in reproductive success and hence, potential for sexual selection in *Ariolimax*.

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APPENDIX

Definitions of Reproductive States in *Ariolimax buttoni*
(Histological data from Westfall 1952)

1. **Immature State**: Reproductive system located anteriorly, close to atrium; ovotestis (=hermaphroditic gland = gonad), white, often transparent; small, thin hermaphroditic duct; spermatheca (=bursa copulatrix = gametolytic gland) flattened, transparent; albumen gland < 20 mm in length, usually smaller than the ovotestes, made up of small, white lobes, close to and alongside ovotestis. Histologically, spermatozoa absent; abundant spermatogonia and spermatocytes, only occasional groups of spermatids; germinal epithelium well-formed, lumen without much connective tissue; fair number of eggs in various stages of development and degeneration. The reproductive system in the immature state resembles that of juvenile slugs.

2. **Intermediate State.** Ovotestis smooth, creamy, solid; greatly expanded hermaphroditic duct; spermatheca, colorless; albumen gland white or sometimes yellow, appearance as immature state. Histologically, cross section with sperm predominant in ovotestes, characteristically arranged in orderly fashion around inner periphery of each acinus; sperm massed in hermaphroditic duct; a few eggs in various stages of development; large oocytes protrude from inner walls of the acini; oocytes easily distinguished by large amount of cytosome in proportion to size of distinct, round, clear nucleus; nucleolus of oocyte usually stains darkly; degenerating oocytes fairly common, appear yolky, usually irregular in outline.

3. **Hypertrophied State.** Ovotestis dark, reddish brown with granular appearance; hermaphroditic duct, convoluted and yellow, pushing ovotestis and albumen glands posteriorly, filled with masses of spermatozoa; spermatheca bulges with red fluid; albumen gland very large (> 20 mm), larger than ovotestis, extending posteriorly, filling up much of the body cavity. The one slug taken for dissection while egg-laying was in the hypertrophied state with oviduct distended with eggs and masses of sperm in hermaphroditic duct. Histologically, ovotestis cross section somewhat ragged, acini noticeably shrunken and detached, separated by wide spaces within ovotestis membranes; all stages of spermatogenesis present, although spermatozoa fewer than in intermediate stage and no longer arrayed in strikingly regular fashion of the intermediate state; eggs large, relatively rare; most ova degenerating, apparently left over from spawning. Portion of hermaphroditic duct visible in ovotestis cross sections shrunken, sperm masses visible only in smears under the microscope.

4. **"Old" State**. "Six dehydrated and wrinkled specimens from a moist terrarium which were dissected had atrophied reproductive organs with straight ducts, but in all cases the hermaphroditic gland was a light reddish-brown color. In three of these specimens the spermatheca seemed white and cloudy. Smears on microscope slides showed that the spermathecae were filled with spermatozoa" (Westfall 1952, p. 46).

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A review of mating behavior in slugs of the genus *Deroceras* (Pulmonata: Agriolimacidae)*

Heike Reise

State Museum of Natural History Görlitz, PF 300 154, 02806 Görlitz, Germany, Heike.Reise@smng.smwk.sachsen.de

Abstract: The genus *Deroceras* Rafinesque, 1820 (the largest genus of terrestrial slugs) shows a high diversity of penis morphologies and mating behaviors. The function of most of the appending external and internal penial structures, some of them truly bizarre, is largely unknown. This paper reviews mating behavior and reproduction, based on data on 16 species from the literature and from unpublished observations. I analyze patterns common to all *Deroceras* species and differences among species. The general mating pattern consists of a long courtship with mutual stroking with a sarcobelum, a sudden penis eversion, and external sperm exchange (copulation). I distinguish also precourtship and withdrawal phases. Sperm exchange is usually very quick but, in a few species, occupies a considerable proportion of the total mating duration. Mutual sperm exchange is the rule. Species differences involve the durations of certain mating phases, presence and nature of initial trail following, nature and intensity of stroking (including the degree of contact with the sarcobelum), aggressiveness of courtship behavior, and the timing of the penial gland eversion. I hypothesize that the radiation of mating behaviors and associated structures has been driven by an arms race resulting from conflicting interests of mating partners over sperm donation and use. This could also have increased the rate of speciation in *Deroceras*. There are indications of the presence of sperm competition and conflicting interests between mating partners: individuals mate repeatedly, can store and digest sperm, and simultaneously use sperm from different mating partners for fertilization. Some details of mating behavior also indicate conflict. The timing of the penial gland eversion after sperm exchange suggests a manipulation akin to the role of love darts in helicid snails. Finally, some recommendations for studying mating behavior in *Deroceras* are given.

Key words: courtship, genital morphology, partner manipulation, sexual conflict, simultaneous hermaphrodite

Deroceras Rafinesque, 1820 is the largest genus of terrestrial slugs (over 100 known species), and comprises the major part of the slug family Agriolimacidae (Wiktor 2000). It is Holarctic with most species restricted to the Palaearctic, although a few synanthropic species have been introduced to most other continents. The widespread pest species have been comparatively well studied. However, there are many species with apparently small geographic ranges, and for a number of species not much more than the morphology and type locality is known. There is no well-supported phylogeny of *Deroceras* available except for the separation of six species into the subgenus *Liolytopelte* Simroth, 1901.

Deroceras slugs are externally rather uniform (each species has some externally identical congeners), and most of the internal anatomy also varies very little among species. Almost the only species-specific characters are provided by the penial morphology: there is a wide variety of appending and internal structures (side pockets, glands, folds, pilasters, etc.), but their functions are largely unknown.

The diversity of penial morphologies is accompanied by a diversity of mating behaviors, and even sibling species can differ considerably (Gerhardt 1935, Reise 1995, 2001, Wiktor 2000). I review here the mating behavior of *Deroceras* and indicate which patterns are consistent in all species and which vary among or within species. The elaborate mating behavior and the diversity of penial structures, including rather extravagant and bizarre structures, caused Reise (2001) to hypothesize that the diversity of mating behaviors and associated genital structures is driven by an evolutionary arms race between male and female functions in these simultaneous hermaphrodites.

The background to this hypothesis is that at least some species of *Deroceras* are able to self-fertilize and/or to mate repeatedly (Rymzhanov and Schileyko 1991, Rymzhanov 1994, Reise 1996, 1997, 2001, Lebovitz 1998, Wiktor 2000 and references he cites on p. 375). Moreover, they may simultaneously use sperm from different mating partners for fertilization of a single clutch (H. Reise, B. Zimdars, M. Scheibe, J. Sauer, and C. Matthieu, unpubl. obs. on *Deroceras panormitanum* (Lessona and Pollonera, 1882)). A receiver might thus not use the donor's sperm and instead use sperm from another (earlier or later) donor or its own sperm to fertilize its eggs (unused ejaculates may be digested in the bursa copulatrix). It is even possible that individuals try not to donate sperm in some matings if it would be better to invest the ejaculate in a higher-quality partner or if there are indications that this partner would only digest the ejaculate (Leonard 1991, Michiels 1998). A sexual conflict could arise if partners attempt to avoid one of the sexual roles (male or female) or if sperm donors can manipulate their partners to use this batch of donated sperm to fertilize more eggs. Counter-adaptations would lead to an evolutionary arms race; this might drive rapid diversification and the development of bizarre genital structures and mating behaviors. Evolutionary arms races driven by sexual conflict have been convincingly shown in gonochorists (Rice 2000), and Leonard (1990, 2006), Michiels (1998), and Michiels and Koene (2006) have proposed that sexual conflict may also be strong in simultaneous hermaphrodites. Convincing evidence for an arms race between sperm-donating and sperm-receiving functions in simultaneous hermaphrodites comes from a comparative analysis of love-dart shooting and receiving organs in helicid snails (Koene and Schulenburg 2005). There are also indications of intraspecific coevolution of male and female reproductive traits in the terrestrial snail *Arianta arbustorum* (Linnaeus, 1758) (Beese *et al.* 2006).

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My review is based on data on mating behavior from 16 species (Table 1). These data include my own published and unpublished observations as well as descriptions by others. The published descriptions vary considerably in quality. Some are based on single or very few chance observations in the field and provide little information. Some others do not specify sample sizes. My own unpublished observations (and those of M. Benke and I. Schulze) cited in this paper are based on laboratory observations of the mating behavior of wild-collected or laboratory-bred individuals. Animals were kept isolated for a few days prior to being put together and then kept under at least periodic observation for a few hours until they did or did not start to mate; thus my observations often include early precourtship behavior. In all species at least some matings were video-recorded.

Comparisons are hampered by inconsistent or unclear definitions of mating phases (see section on general mating pattern) and by uncertain species identities; these have also led to misunderstandings between authors. A particularly good example is Carrick's (1938) description of the mating behavior of *Deroceras agreste* (Linnaeus, 1758) (probably what we now know as *D. reticulatum* (Müller, 1774), see below), based on one field observation in Scotland. The author seems to have misunderstood the sarcobelum as the penis, and he reported that it was inserted into the partner's genital aperture. Consequently, he interpreted the entire courtship as copulation and saw discrepancies with the copulation time given for this species by Taylor (1902-1907). He may possibly have been confused by Heath's (1916) description of the mating behavior of *Ariolimax californicus* (Gould *in* A. Binney, 1851), an arionid slug with penis intromission. The genus *Deroceras* was called *Agriolimax* Mörch, 1865 at this time, and a misspelling in the reference list implies that he confused the similar generic names and thought to see what he expected.

The commonest example of uncertain species identity is that in older papers *Deroceras reticulatum* was usually not distinguished from *D. agreste*, so that it is unclear which

Table 1. Sources of data on mating in *Deroceras*. (*: species identity uncertain).

Subgenus *Deroceras* s.s.	
D. agreste (Linnaeus, 1758)	Gerhardt 1933, 1934*, H. Reise, unpubl. obs. (1 mating)
D. fatrense Mácha, 1981	Reise, unpubl. obs. (≥12 matings)
D. gorgonium Wiktor *et al.*, 1994	Reise *et al.* 2007
D. laeve (Müller, 1774)	Karlin and Bacon 1961*, Rymzhanov 1994*, Barker 1999*
D. lombricoides (Morelet, 1845)*	Simroth 1891, Castillejo *et al.* 1989
D. nitidum (Morelet, 1845)	Castillejo *et al.* 1989
D. panormitanum (Lessona and Pollonera, 1882)	Gerhardt 1939, Quick 1960, Webb 1961, 1965, Barker 1999, Reise and Hutchinson 2001b, Benke *et al.* 2005, Benke 2006, H. Reise, M. Scheibe, J. Sauer and C. Matthieu, unpubl. obs. (≥60 complete matings)
D. planarioides (Simroth, 1910)	Gerhardt 1939*
D. praecox Wiktor, 1966	Reise 1995, unpubl. obs. (≥29 matings)
D. rethimnonensis de Winter and Butot, 1986	Wiktor 1994
D. reticulatum (Müller, 1774)	Simroth 1885, Gerhardt 1933*, 1934, Wiktor 1960, Karlin and Bacon 1961, Webb 1961, 1965, Nicholas 1984, Barker 1999, H. Reise, unpubl. obs. (≥2 matings)
D. rodnae Grossu and Lupu, 1965	Reise 1995, 1997, unpubl. obs. (≥33 matings)
D. sturanyi (Simroth, 1894)	Gerhardt 1936*—as "*D. laeve*", Kosińska 1980, Rymzhanov 1994, H. Reise and C. Natusch, unpubl. obs. (6 matings)
D. turcicum (Simroth, 1894)	Gerhardt 1935*—as "*Deroceras* aff. *turcicum*", H. Reise, unpubl. obs. (≥7 matings)
Subgenus *Liolytopelte*	
D. bureschi (Wagner, 1934)	Wiktor 1983, 2000
D. caucasicum (Simroth, 1901)	Rymzhanov and Schileyko 1991

species were observed (Wiktor 2000). Gerhardt's (1933, 1934, 1936, 1939) valuable descriptions of the mating behavior of several *Deroceras* species were hampered by uncertain species determinations (Gerhardt 1934, 1939, Wiktor 1960). In 1933, he published descriptions for "*Agriolimax agrestis*" and "*Agriolimax laevis*", but later corrected their identities to "*Deroceras reticulatum*" and "*Deroceras agreste*", respectively (Gerhardt 1934), which is how I will refer to his 1933 descriptions. Then in 1936 Gerhardt described the mating behavior of a species that he thought to be the real *Deroceras laeve* (Müller, 1774). However, he later expressed some uncertainty about the species identity (Gerhardt 1939). It seems probable that he was indeed wrong again: all details that Gerhardt (1936) provided about his *D. laeve*—time of occurrence, body color, sarcobelum shape and its use during courtship, bulbous shape of everted penial mass, and unusually long copulation—fit very well with the externally hard-to-distinguish *D. sturanyi* (Simroth, 1894), a species of which malacologists were hardly aware at that time and with which *D. laeve* has often been confused (Quick 1960). Later descriptions of matings of *D. laeve* with which one might compare are sparse: Karlin and Bacon (1961) repeated Gerhardt's (1936) statement that its courtship is similar to that of *D. reticulatum* but stress that the partners have less intimate contact. However, they do not mention copulation, which they probably would have done had they observed it. Barker (1999) just repeated information provided by Gerhardt (1936) and Karlin and Bacon (1961), so one has to wonder to what extent Barker observed the mating of *D. laeve*. Rymzhanov's (1994) description of the mating behavior of *D. laeve* from Kazakhstan differs in almost every aspect from the earlier papers. The origin that Rymzhanov (1994) proposed for the aphallic individuals in his population (apophallation) differs from what we know of their origin in Europe, so Kazakh *D. laeve* may well be a distinct species. Thus, although four publications claim to describe the mating behavior of *D. laeve*, there are no reliable data. In this paper I will consider the species studied by Gerhardt (1936) as *D. sturanyi* and refer to the one described by Rymzhanov (1994) as "Kazakh *D. laeve*". The papers of Karlin and Bacon (1961) and Barker (1999) will be assumed to pertain to *D. laeve*, because *D. sturanyi* is unknown in North America and New Zealand, but this should be viewed as provisional.

I do not include other genera of the Agriolimacidae because at least some are considerably different in their genital anatomy and thus possibly also in their mating behavior. Besides, almost nothing is known about them. The only published description of mating behavior in another agriolimacid genus concerns a species of *Furcopenis* Castillejo and Wiktor, 1983 (Rodríguez *et al.* 1989), morphologically the most similar genus to *Deroceras*, of which it had formerly been classified as a subgenus.

Casual observations suggest that mating often occurs during early morning, but no one has carried out systematic observations throughout 24 hours, so I do not attempt to review this aspect of mating behaviour. Mating slugs are often observed in the open, but again there are no systematic observations of what proportion of matings in the wild are hidden, for instance under leaves.

GENITAL MORPHOLOGY

There are discrepancies in how to describe the relative position of parts of molluscan genitalia. Sometimes, the parts further away from the genital pore are called "proximal" (*e.g.*, Castillejo *et al.* 1989, Rymzhanov 1994, Reise 1997, 2001, Hausdorf 1998, Barker 1999, Reise and Hutchinson 2001a) and sometimes "distal" (*e.g.*, Quick 1960, Webb 1965, Nicholas 1984, Backeljau and De Bruyn 1990, Reise 1995). Some authors use "anterior" and "posterior", "basal" and "terminal", or other terms relating to the approximate positions of the organs in the animal at rest and their orientations (*e.g.*, Mead 1943, Webb 1961, Sirgel 1973, Rähle 1998, Tompa 1984, Wiktor 2000). However, as not all sections of the genital tract are orientated anterior-posterior in animals at rest, I will use the terms proximal and distal and apply the first definition, which is that used in medicine: parts of the genitalia nearer to the genital pore are further away from the body centre (when not everted) and thus distal. Parts further away from the genital pore, and thus nearer to the body centre, are proximal (Fig. 1).

In the genital tract of *Deroceras* (Fig. 1), the penis has a more or less sac-like shape, but can consist of one or more chambers and may have side pockets and diverse appendages. In at least one species, *D. laeve*, the penis is often

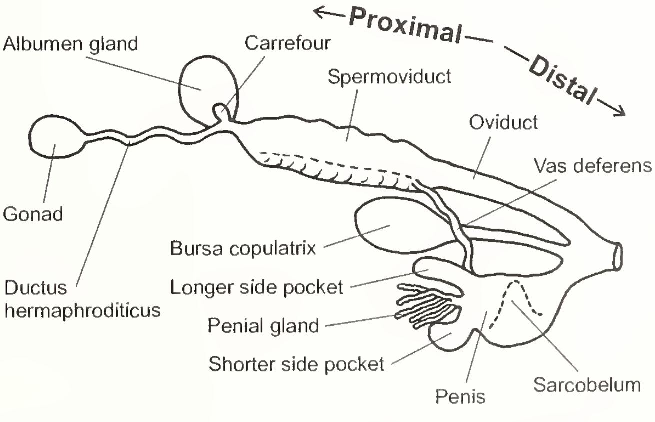

Figure 1. Schematic genital tract of *Deroceras panormitanum* [after fig. 5.1 in South (1992)].

reduced (hemiphallic) or entirely lacking (aphallic) (Simroth 1884, Wiktor 1973, 2000, Tompa 1984).

Most species have a more-or-less finger-like penial gland appending somewhere near the proximal end of the penis. This is also called the trifid or penial appendage (*e.g.*, Quick 1960, Runham and Hunter 1970, Runham 1978) or flagellum (*e.g.*, Simroth 1885, Gerhardt 1933, 1935, Wiktor 1960, Webb 1961); it may or may not be homologous to the flagellum of helicid snails (Sirgel 1973, Nicholas 1984, Hausdorf 1998). The name "penial gland" seems justified because there are indications for secretory activity (Sirgel 1973, Nicholas 1984, Benke et al. 2005, Benke 2006). Interspecifically, the penial gland varies widely in size, can be branched or unbranched, and lobed or smooth (Fig. 2). In some species, particularly *Deroceras gorgonium* Wiktor *et al.*, 1994 (Fig. 2F), it is extremely large and tree-like. The number of branches can vary intraspecifically (Wiktor 2000, Benke 2006).

The lumen of the penis also contains diverse structures. The most important is the sarcobelum (or stimulator; "Reizkörper" of earlier German authors), located in the distal, swollen part of the penis. It is a conical or tongue-like structure, solid but with a central blood sinus, and consists of muscle, glandular, and connective tissue cells in a collagen matrix (Nicholas 1984). The sarcobelum plays an important role during courtship when it is pushed outside the genital orifice by the eversion of the distal part of the penis (the sarcobelum itself does not evaginate). Its surface has longitudinal ridges with a strongly ciliated epithelium, and there are gland cells in the sarcobelum and the surrounding inner penial wall (Sirgel 1973, Els 1978, Nicholas 1984). This and its use during courtship suggest that this organ transfers secretions onto the mating partner. Shape and size of the sarcobelum vary considerably among species (Fig. 3). Species of the subgenus *Liolytopelte* have a calcareous plate at the base of the sarcobelum (Fig. 3D).

The walls of other penial protuberances have also been reported to contain glands (Wiktor 2000). However, the function of these and most other penial structures is largely unknown.

The sac-like bursa copulatrix (also called "spermatheca") opens, via the bursa trunk, into the genital atrium (Nicholas 1984) or base of the penis (Hausdorf 1998, Wiktor 2000). It is a lytic organ and digests excess sperm as well as some other secretions (Nicholas 1984, Tompa 1984), probably including other components of ejaculates.

GENERAL MATING PATTERN

The mating pattern of *Deroceras* consists of four main phases (Fig. 4).

(i) Precourtship phase: the partners encounter and investigate each other.

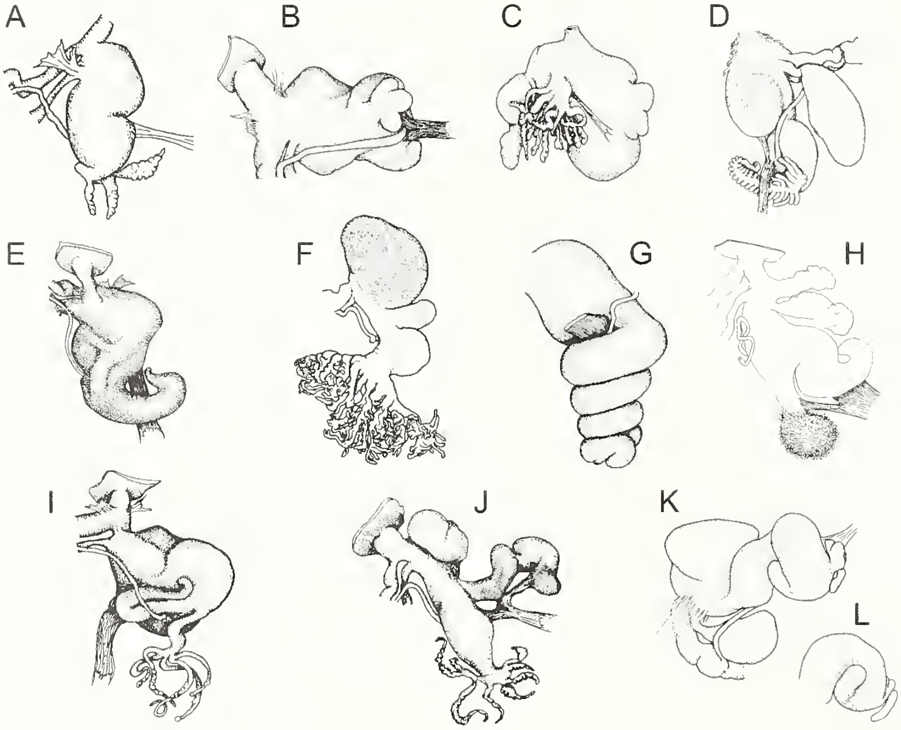

Figure 2. Penis diversity in *Deroceras*. A, *D. reticulatum*. B, *D. minoicum* Wiktor *et al.*, 1994. C, *D. ikaria* Reischütz, 1983. D, *D. christae* Rähle, 1998. E, *D. adolphi* Wiktor, 1998. F. *D. gorgonium*. G, *D. helicoidale*. H, *D. glandulosum* (Simroth, 1904). I, *D. giustianum* Wiktor, 1998. J, *D. oertzeni* (Simroth, 1889). K, L, *D. praecox*. L, pocket at proximal end of penis—different perspective of same specimen as K. Sources of drawings: A, H, Wiktor 2000; B, Wiktor *et al.* 1994; C, J, Wiktor 2001; D, G, Rähle 1998; E, I, Wiktor 1998; F, Reise *et al.* 2007; K, L, Reise and Hutchinson 2001a. Reproduced by permission of the Museum and Institute of Zoology of the Polish Academy of Sciences (A, D, H), the Museum of Zoology Dresden (B, G, E, I), the Natural History Museum of Crete (C, J), Springer (F), The Association of Polish Malacologists (K, L).

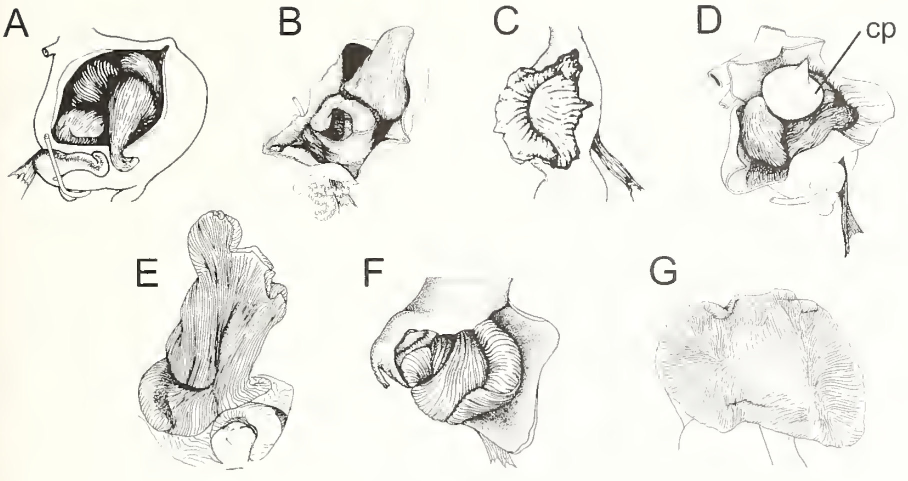

Figure 3. Variability of sarcobelum shapes in *Deroceras*. Conical sarcobelum: A, *D. giustianum* Wiktor, 1998. B, *D. rethimnonensis*. C, *D. laeve*. With calcareous plate at base of sarcobelum (cp): D, *D.* (*Liolytopelte*) *caucasicum*. Flat sarcobelum: E, *D. rodnae* from SE Poland. F, *D. subagreste* (Simroth, 1892). G, *D. bistimulatum* Wiktor, 2000 (sarcobelum consisting of two lobes; here protruded through the genital opening). Sources of drawings: A, Wiktor, 1998; B, Wiktor 2001; C, D, F, G, Wiktor 2000. Reproduced by permission of the Museum of Zoology Dresden (A), the Natural History Museum of Crete (B), the Museum and Institute of Zoology of the Polish Academy of Sciences (C, D, F, G).

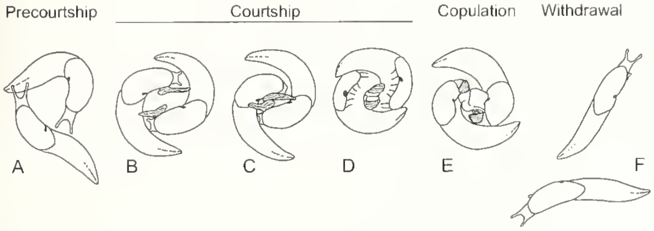

Figure 4. General mating pattern of *Deroceras* [after Reise (1995)].

(ii) Courtship phase: both partners have their sarcobelum protruded from the genital opening and assume a position with their genital pores facing each other, forming a circle or yin-yang configuration.

(iii) Copulation phase: the slugs evert their penes, entwine them, and mutually transfer the ejaculates from penis to penis (there is no intromission).

(iv) Withdrawal phase: the penes are retracted together with the attached sperm masses.

I consider the beginning of precourtship as the moment when two slugs start to show clear signs of interest: investigating each other with tentacles or mouth, circling, or trail following. I refrain from the synonymous term "recognition phase" (*e.g.*, Reise 1995) because this implies too restricted a function. I define courtship as starting when both partners have everted their sarcobela. The separation of precourtship and courtship seems reasonable in the majority of species in which the behaviors during these two phases clearly differ. However, there are species with less clear-cut separations: for example, in *Deroceras gorgonium* the partners may not evert their sarcobela at roughly the same time and they may retract them repeatedly (Reise *et al.* 2007). For this reason, and to enable comparisons with other authors, I also use the term "precopulatory phase", meaning precourtship + courtship. Barker (1999) occasionally used this term without clearly defining it.

I define the beginning of copulation as when penis eversion starts (excluding the earlier partial eversion of the distal penis that protrudes the sarcobelum). Copulation ends and withdrawal starts when the genitalia lose contact with the partners. Withdrawal ends when the genitalia are fully retracted into the body (but not necessarily into the original position within the body, which can take much longer: Webb 1961, Nicholas 1984). Behavior related to mating may continue after withdrawal: during mating, particularly during copulation, the partners secrete abundant mucus, which covers the mating substratum (Simroth 1885, Gerhardt 1933, Wiktor 1960, 2000, Karlin and Bacon 1961, Kosińska 1980, Reise 1995), and this is often eaten by one or both partners after withdrawal (Kosińska 1980, Wiktor 2000). Also, slugs have been observed to lick off penial gland secretion received during copulation (see copulation section).

There are some discrepancies in the distinction of mating phases and their nomenclature by different authors. This complicates interspecific comparisons. For example, Castillejo *et al.* (1989) called the entire mating (except precourtship) "copulation", whereas Rymzhanov and Schileyko (1991) called it (including precourtship) "courtship", as apparently did Barker (1999). At one point in his paper Rymzhanov (1994) applied a Russian term for "mating play" to the entire mating, as do Rymzhanov and Schileyko (1991) but at another point he restricted this term to only the precopulation phase.

Often, particularly in older publications, mating is divided into only two phases: the behavior before copulation and the copulation. The first part has been called the nuptial dance (Wiktor, 1960, Pilsbry 1948), Vorspiel (*i.e.*, foreplay: Simroth 1885, 1891, Gerhardt 1933, 1935, 1939, 1940) or courtship (Webb 1961, Tompa 1984). It is often unclear whether the descriptions of courtship include only courtship behavior in my strict sense or also precourtship. Often little attention has been paid to this precourtship phase, either because the behavior was not recognized as early mating, or because observations started only at courtship (usually the case with field observations). However, in some cases precourtship was clearly considered as a first part of courtship, as by Karlin and Bacon (1961) who used the term "positional movements", or it was distinguished as a separate "recognition" phase (Kosińska 1980, Reise 1995, 1997, Wiktor 2000). This is then followed by the "stimulation phase", "mating dance" or "excitatory movements" (courtship in my strict sense; Karlin and Bacon 1961, Wiktor 2000) or "courtship" and "pre-copulation" (Kosińska 1980). Rymzhanov (1994) distinguished three different precopulatory phases: recognition (for the first, short mutual investigations), following (for apparent trail-following behavior, but including some time when sarcobela are already everted), and circling.

The copulation, also called coition (Karlin and Bacon 1961, Webb 1961) or Begattung (Gerhardt 1933, 1936), has also not been clearly defined. While almost all authors seem to agree that copulation starts when the major parts of the penes begin eversion and entwine, I know of no publication clearly defining the ending of the copulation and withdrawal phases. In only a few papers has withdrawal been distinguished as a phase (Reise 1995, 1997, Wiktor 2000); it was called "postcopulation" in *Deroceras sturanyi* and Kazakh *D. laeve* (Kosińska 1980, Rymzhanov 1994).

Matings are sometimes broken off, primarily during the precourtship or early courtship phases (Kosińska 1980, Rymzhanov 1994, Reise 1995, Wiktor 2000, M. Benke, pers. comm.); this suggests that the function of the long courtship is not mate choice but there could be an influence of courtship on sperm exchange or on its subsequent use. A retreat from courtship into precourtship behavior is also possible; that is, one or both slugs retract their sarcobelum but evert it again later. This seems to happen to different degrees in different species (see comments above on *Deroceras gorgonium*).

Sometimes more than two slugs are involved in precourtship and/or courtship (Simroth 1885, Gerhardt 1933, 1935, Wiktor 1960, Karlin and Bacon 1961, Kosińska 1980, Rymzhanov and Schileyko 1991, Reise 1995, unpubl. obs.). Either all slugs (usually three, but Rymzhanov and Schileyko [1991] saw up to seven) start mating behavior more or less simultaneously, or one individual is attracted by a mating couple. Rymzhanov and Schileyko (1991) stated that the precourtship phase is omitted in such cases, and that participating individuals had usually mated already, but Reise *et al.* (2007) observed trail following with alternating participation. The participation of additional individuals in a courtship can lead to apparent confusion and seems to delay the mating process (Rymzhanov and Schileyko 1991, H. Reise, unpubl. obs.). Never were more than two slugs observed to be involved in a copulation (Karlin and Bacon 1961, H. Reise, unpubl. obs.); either courtship was broken off by all slugs (and two slugs might start again later) or one partner would leave earlier (H. Reise, unpubl. obs.) or would not participate in copulation (Simroth 1885).

Individuals will usually mate again after a few days (H. Reise, unpubl. obs.). However, Rymzhanov and Schileyko (1991) and Rymzhanov (1994) observed that *Deroceras sturanyi* would mate only twice, and that in *D. caucasicum* (Simroth, 1901) the third and fourth courtships would not lead to copulation. This does not agree with my own observations on various other species of *Deroceras*; this discrepancy might have to do with species differences or with methodology. I found that animals will remate in the laboratory more than twice if isolated for several days between matings, but they stop showing interest in mating at a later stage of adulthood, irrespective of whether they have mated already or not. Rymzhanov and Schileyko (1991) used field-collected specimens so they did not know the slugs' ages and possibly not their full mating histories.

TIMING

Even allowing for uncertain or differing definitions of each phase in different publications, species clearly differ considerably in the absolute and relative durations of mating phases (Table 2). These timing differences can act as efficient precopulatory isolation mechanisms (Reise 1995, Wiktor 2000). For example, individuals from allopatric populations of *Deroceras rodnae* Grossu and Lupu, 1965 and *Deroceras praecox* Wiktor, 1966 court with each other in the laboratory, but there is no overlap of the species-specific durations of courtship. The slug with the shorter courtship phase (*D. praecox*) proceeds to the copulation phase (*i.e.*, everts the penis) when its partner is still at early courtship. Because there is no receptive partner (*i.e.*, not another everted penis to entwine with), the *D. praecox* individual retracts its penis together with its own ejaculate, and mating is broken off (Reise 1995).

In most species in which mating has been observed, the courtship phase (or precopulatory phase) lasts much longer than the copulation and takes up most of the mating. The shortest known courtships, in *Deroceras reticulatum* and *D. praecox*, take about 15-20 min (but courtship can take longer in both species) and the longest courtship takes more than 7 h (*D. gorgonium*; the entire precopulatory phase may take 9 h: Reise *et al.* 2007).

The copulation is usually very brief compared to the

Table 2. Duration of different mating phases in *Deroceras.*

Species	Precourtship	Courtship	Copulation	Withdrawal	Reference
D. agreste		>48 min	30 s		H. Reise, unpubl. obs.
		>20->50 min	>60 s		Gerhardt 1933, 1934
D. gorgonium	95-276 min	145-434 min	18-25 s		Reise *et al.* 2007
D. laeve			up to 60 min		Barker 1999
Kazakh *D. laeve*		60-70 min (including precourtship?)	30-50 s	long (apophallation)	Rymzhanov 1994
D. lombricoides		up to >60 s	long		Simroth 1891
D. panormitanum			>10-15 min		Webb 1961
		>20-30 min	>10-15 min		Barker 1999
		up to >45 min	<3-12.5 min	usually 3-5 min	Gerhardt 1939
		ca. 50-80 min	ca. 3-12 min		H. Reise, unpubl. obs.
	0-28 min (mean = 12.5 min)	44-107 min (mean = 66.7 min)	0.8-9.5 min (mean = 2.7 min; without penial gland eversion)	0.7-5.1 min (mean = 2.6 min)	Benke 2006
D. planarioides		ca. 60 min	≤10 s until start of penis retraction		Gerhardt 1939
D. praecox		20-60 min	30-60 s		Reise 1995
D. rethimnonensis		ca. 30 min			Wiktor 1994
D. reticulatum		30->90 min	<15 s		Simroth 1885
		>45 min	28-49 s		Gerhardt 1933, 1934
		up to >70 min	ca. 30 s		Wiktor 1960
		30-almost 120 min	<60 s		Karlin and Bacon 1961
		15-36 min	"seconds, or at most a few minutes"		Webb 1961
		65 min	ca. 30 s		Nicholas 1984
		30-75 min	ca. 30 s		Barker 1999
D. rodnae		95-200 min	30-60 s		Reise 1995
D. sturanyi	usually >10 min	usually 30-40 min (up to 70 min)	"a few hours"	"some time"	Kosińska 1980
	15-120 min	52-169 min	60-144 min	1-70 min	H. Reise and C. Natusch, unpubl. obs.
			>19-71 min		Gerhardt 1936
		26-71 min	140-168 min		Rymzhanov 1994
D. turcicum		longer than *D. reticulatum*	very fast		Gerhardt 1935
		up to >240 min	ca. 20 s		H. Reise, unpubl. obs.
D. caucasicum	90-210 min*		3-4 min		Rymzhanov and Schileyko 1991

* refers to precourtship and courtship

precopulatory phase, and penis eversion starts rather suddenly, often almost explosively. At one extreme, copulation lasts only about 20 s in *Deroceras turcicum* (Simroth, 1894) (Gerhardt 1935, H. Reise, unpubl. obs.) and perhaps only 10 s in *Deroceras planarioides* (Simroth, 1910) (exact end of copulation unclear; Gerhardt 1939). However, copulation can last considerably longer and, at least in one species, can even take longer than the courtship (*D. sturanyi* has a 26-71 min courtship and a 60-168 min copulation: Kosińska 1980, Rymzhanov 1994, H. Reise and C. Natusch, pers. obs.).

The durations depend also on temperature (Wiktor 1960, 2000, Karlin and Bacon 1961, Kosińska 1980, Rymzhanov and Schileyko 1991) and possibly humidity and light regime (Rymzhanov and Schileyko 1991). There is also considerable intraspecific variability (Table 2). Karlin and Bacon (1961) observed that even in couples of *Deroceras reticulatum* mating at the same time, the duration of precourtship varied by a factor of three.

PRECOURTSHIP PHASE

The initial mating behavior, lasting until eversion of both sarcobela, is the phase with the least published information about it, because casual observations are usually made when mating has already started. Probably in all species the partners initially investigate each other with their tentacles and mouth and eat the partner's body mucus (Kosińska 1980, Rymzhanov 1994, Reise 1995, Wiktor 2000).

Many species show some degree of trail following, which can constitute a major part of the precourtship phase. This behavior has been described as simple directional following of one slug along the mucus trail of another to catch up with a potential mating partner (Wareing 1986, Wiktor 2000) and has sometimes also been called a chase (Gerhardt 1933, Webb 1961). However, my own observations indicate that trail following during the recognition phase is a complex behavioral pattern involving the active participation of both partners. Usually the partner following keeps very close to the leader. In *Deroceras panormitanum* and other species with pronounced trail following, the tail is flattened laterally and becomes a flag-like structure (Fig. 5); it is slightly lifted up above the ground, and waves side-to-side, either in front of the follower's head or between its tentacles, thus contributing to the occasional contacts between tail and tentacles. If the follower falls behind, the leader seemingly tends to wait for it, tail waving. It would be interesting to conduct experiments in the dark to test whether tail waving acts as a visual stimulus, but it might also emit a chemical attractant.

Trail following has been observed in *Deroceras panormitanum* (H. Reise, unpubl. obs., Benke 2006, but see below), *D. gorgonium* (although little pronounced, Reise *et al.* 2007) and *D. sturanyi* (Kosińska 1980, Rymzhanov 1994). It was described as a regular component of mating in *D. reticulatum* by Gerhardt (1933), Quick (1960), and Wareing (1986), but Webb (1961) and Barker (1999) indicated that it occured only occasionally. I observed that it did not occur in *D. rodnae* and *D. praecox* (Reise 1995, 1997); Barker (1999) stated that it did not occur in *D. laeve*; but Rymzhanov (1994) did describe it in Kazakh *D. laeve*. The fact that it was not mentioned by Gerhardt (1936, 1939) for *D. planarioides* and *D. sturanyi* nor by Nicholas (1984) for *D. reticulatum* is more difficult to interpret.

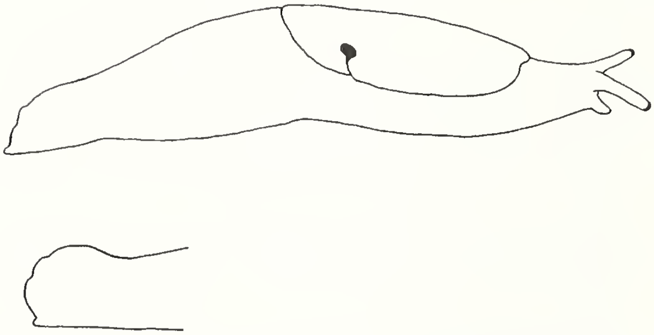

Figure 5. Tail enlargement during trail following in *Deroceras panormitanum*.

The idea that trail following serves for catching up with a potential mate implies that the follower is the more active, mating-initiating partner. However, the tail waving indicates that the leader's role can be much more interactive than has been assumed. Moreover, in *Deroceras panormitanum* almost always, and in *D. gorgonium* usually, it is the leader that extrudes its sarcobelum first (Benke 2006, Reise *et al.* 2007, H. Reise, unpubl. obs.). Moreover, later in courtship, at least in *D. gorgonium*, this trail-leading partner is also the first to exhibit each successive behavioral pattern. In Kazakh *D. laeve*, the leader is the first to touch the partner during courtship and to retract its penis after copulation (Rymzhanov 1994). Thus, if there is a partner with an initiating role, it is probably the leading slug. However, this might differ interspecifically, because the follower everts its sarcobelum first in *D. caucasicum* (Rymzhanov and Schileyko 1991) and *D. sturanyi* (Rymzhanov 1994).

By the end of precourtship, the partners form an open circle with their heads towards the partner's tail and genital pores pointing towards the inside of the circle, and they often begin circling. In trail-following couples, this position is reached by the leading slug finally crawling in a bow back towards the follower, usually towards the latter's tail. This is often when the second partner or both partners evert the sarcobelum and start courtship.

The behavior and duration of the precourtship phase vary considerably, not only between species (Table 2) but also within species, probably owing to variation in the motivation to mate (Benke 2006, Reise *et al.* 2007, H. Reise, unpubl. obs.). Slugs in which isolation is likely to have generated a high motivation to mate tend to abridge the precourtship phase and may even move on to courtship soon after first contact (see below). This supports the suppositions that precourtship serves to assess a partner's readiness to mate (Wiktor 2000) and that some behavioral patterns might also aim at motivating a partner that does not show initial interest. A role in species recognition is also possible (Wiktor 2000).

Intraspecific variability might be the reason for some discrepancies in descriptions of precourtship by different authors. For instance, trail following usually occurs in *Deroceras sturanyi* but can be skipped by some couples (Rymzhanov 1994). Probably more often, however, these differences may be caused by incomplete observations or differing opinions about whether a behavior should be considered part of mating. In *D. panormitanum*, for example, Webb (1961, 1965) and Barker (1999), based on observations of unspecified numbers of couples from France and New Zealand, reported that mating starts with circling (which occurs after trail following) and Gerhardt (1939, based on about 30 couples from Wales) even expressly stressed that there was no trail following. However, in the more than 150 matings of *D. panormitanum* from the UK, Belgium, the Netherlands, and Germany that I and my co-workers have watched, this behavioral pattern was almost always present, although occasionally very brief.

COURTSHIP PHASE

Courtship begins when both partners protrude their sarcobela; this is usually a rapid process. Eversion happens simultaneously (Wiktor 2000) or one soon after the other (Wiktor 2000, H. Reise, unpubl. obs.). In *D. panormitanum* this is a rather fixed process: as described above, the slug leading the trail following turns back towards the follower, and they then form a circle. Most often, the first slug everts at the moment of turning back, and the second slug everts soon after formation of the circle. However, sometimes the second sarcobelum has everted shortly before the circle is formed, or sometimes the first sarcobelum everts shortly after circle formation (Benke 2006, H. Reise, unpubl. obs.). In contrast, sarcobelum eversion in *D. gorgonium* is quite variable: often one slug everts long before the other and may retract repeatedly. However, eversion can also be almost simultaneous in this species, and the temporary retractions do not always occur. This causes a highly variable duration of the precourtship (95-276 min) and courtship phases (145-434 min) in this species. However, the duration of the entire precopulatory phase varies much less than each component phase (about 7-9 h); so a long precourtship is followed by a short courtship and *vice versa* (Reise *et al.* 2007).

Kosińska (1980) reported that the sarcobela are "generally, although not always" everted during courtship of *Deroceras sturanyi*. It is unclear whether she meant that one or both partners might not evert the sarcobelum at all during courtship (which would be unique amongst *Deroceras*), or whether it had already been everted earlier.

Stroking the partner with the sarcobelum, or at least apparent efforts to use it to touch the partner, are the most prominent aspects of courtship. As soon as the sarcobela are everted, the slugs direct them towards their partners, and by then they have formed a configuration with their genital pores facing towards one another; often this is a circular configuration. Sooner or later, the partners position themselves more and more into a yin-yang position: each head is at the partner's side and the genital openings thus lie close to each other (Fig. 4B-C). A tight yin-yang is the position for copulation. In all species, slugs get progressively closer during courtship and stroking intensity progressively increases but stops just before copulation.

There are large interspecific differences in the duration of the courtship phase (see section on timing), the intensity and speed of circling, the position of the partners towards each other, the way of stroking, and to what extent there is an aggressive component. I now discuss these in turn.

All species show some circling during most of the courtship (Barker [1999] stated that there is no circling in *Deroceras laeve*, but see the introduction section about my doubts). Circling is almost always clockwise and more or less around a central point which hardly moves. If each partner follows the other's tail, they form a circle; if each crawls towards the other's right side, they assume the yin-yang position; in both cases I term the movement "circling". Occasionally, one partner may leave the position and circle one or two turns around its own axis or around a slower partner, but it will always return into the original circle or yin-yang configuration (Wiktor 1960, Rymzhanov and Schileyko 1991, H. Reise, unpubl. obs.).

Most species start with a circle and then slowly change towards yin-yang, as in *Deroceras reticulatum* (Gerhardt 1933, Webb 1961, H. Reise, unpubl. obs.), *D. sturanyi* (Kosińska 1980), and *D. caucasicum* (Rymzhanov and Schileyko 1991). Others are in the yin-yang position from a very early stage (*D. rodnae*, *D. praecox*, *Deroceras fatrense* Mácha, 1981: Reise [1995, 1997, unpubl. obs.]). *Deroceras gorgonium* seems unusual in that almost the entire courtship consists of two alternating behavioral patterns: individuals wave their sarcobela whilst remaining stationary (see below) and then circle half a revolution (so that each slug ends up in its partner's former position).

The speed of circling generally decreases later in courtship. Kosińska (1980) distinguished two phases of courtship in *Deroceras sturanyi*: (i) a "quick circular dance" in a larger circle, with the mouth and sarcobelum touching the partner's tail, lasting usually for 20-30 min, but sometimes more than 1 h and (ii) a "slow circular dance" in a smaller circle, lasting about 10 min and occasionally interrupted by 40-65 s bouts in the yin-yang position when their sarcobela and mouths touch the area around the other's genital opening. During the "quick dance", the partners needed usually 20-60 sec to complete one circle, and during the "slow dance", 60-90 s. The decrease of circling speed in *D. sturanyi* was also noted by Gerhardt (1939) and Rymzhanov (1994), although Rymzhanov's timings are contradictory: first 80-95 s per revolution, and later 68-75 s. Specimens of Kazakh *D. laeve* take 1.5-2.5 min during early courtship and 4.5-5 min later (Rymzhanov 1994). Also in *D. caucasicum*, circling is faster at the beginning (2 min per circle) than later (10-12 min) (Rymzhanov and Schileyko 1991).

All sarcobela, particularly the larger ones, are very maneuverable organs and seem adapted for touching the partner and for transferring a secretion onto its body. It would make adaptive sense also if they had a chemosensory function in assessing the physiological state of the partner, but there is no evidence for this. As there is an enormous diversity of sarcobelum shapes and sizes (Fig. 3), it is not surprising that also their use varies considerably. Species with large, flat, tongue-like sarcobela appear particularly efficient at transferring secretions. Such sarcobela are usually laid flat onto the partner and stroked along its body. The large area of such sarcobela and their extremely flexible movements ensure that much body surface is covered. *Deroceras fatrense* and *D. rodnae* stroke mainly the partner's back and side, most often around the mantle, using the underside of the sarcobelum (although some individuals of *D. rodnae* also often use the narrow edge). *Deroceras praecox* strokes mainly the partner's lower flank and the sole using the upper side of the sarcobelum (Reise 1995, 1997, unpubl. obs.). There are many other species that have a flat sarcobelum, but their mating behavior has not been observed. So, it remains to be examined whether large flat sarcobela consistently stroke more intensely and closely than others (but see descriptions of *D. caucasicum* and *Deroceras lombricoides* (Morelet, 1845) below).

The majority of species have conical sarcobela, varying from rather short, stout organs to long, pointed, finger-like ones. The medium-long sarcobela of *Deroceras reticulatum*, *D. agreste*, *Deroceras nitidum* (Morelet, 1845), and *D. sturanyi* are used to stroke the partners during almost the entire courtship, but the limited length and maneuverability permit touching only the facing flank of the partner, and the conical shape does not allow very broad contact. The rather aggressive *D. panormitanum* touches considerably less frequently and less intensely during the first part of courtship than other species with similar sarcobela (see below).

Short bump-like sarcobela such as in some *Deroceras laeve* (Wiktor 2000) surely cannot stroke as well as longer ones and must reach the partner only when it is very close. This is one reason why I suspect that published descriptions of *D. laeve* mating behavior (Gerhardt 1936, Karlin and Bacon 1961) have dealt with different species (see also the introduction section). In Kazakh *D. laeve*, Rymzhanov (1994) seems to have seen more touching with the mouth than with the sarcobelum. Detailed descriptions of the courtship of species with such rudimentary sarcobela would be highly valuable.

At the other extreme, *Deroceras gorgonium* has a very long, slim sarcobelum with a sharply pointed tip. There is almost no body contact during early courtship (which can last for several hours), and the sarcobelum, stretched out perpendicular to the body, merely waves in front of the partner's face. Only later do the partners get closer, but even then for much of the time the sarcobela touch each other or the partner's body just with their tips. We have occasionally observed transfer of secretion droplets via the tip of the sarcobelum (Reise *et al.* 2007). Maybe the sarcobela are so long in this species to bridge the long distance between partners and to apply the secretion, and animals might keep so far apart to avoid receiving the secretion. Secretion droplets have also been observed on the sarcobela of courting *D. caucasicum* (S. Leonov, pers. comm.).

There are a few species for which the stroking behavior is unusual in some way, and reexamination would be desirable. Rymzhanov and Schileyko (1991) describe the mating of *Deroceras* (*Liolytopelte*) *caucasicum* from some introduced Kazakh populations (Wiktor 2000) where the sarcobelum is used for intense stroking but, judging from their published figures, the partners seem to take up the yin-yang position only shortly before copulation. Their figures show a large flat sarcobelum covering rather large parts of the partner's body. However, there are contradictory opinions about whether the sarcobelum is like this (Rymzhanov and Schileyko 1991, Likharev and Wiktor 1980) or conical (Wiktor 2000, S. Leonov, personal communication: photographs of mating couples from the Crimea). This might reflect geographical differences between populations or indicate different taxa.

The sarcobelum of *Deroceras lombricoides* is also flat, but unusually thin, wide and very short (Wiktor 2000). At eversion, it resembles the arionid ligula (Wiktor 2000), and one wonders how this fold-like structure can stroke a partner. However, a huge flat lobe is pressed onto the partner's back during courtship (fig. XI in Simroth 1891, Castillejo *et al.* 1989), so it seems that the bulky, distal part of the penis is everted through the genital pore together with the horseshoe-like fold mounted upon it.

The courtship of *Deroceras* (*Liolytopelte*) *bureschi* (Wagner, 1934) also appears to be unusual, although the scanty description is based on only a single field observation (Wiktor 1983, personal communication). The "inconspicuous" sarcobelum (Wiktor 2000) is so small that it is not even mentioned in an earlier anatomical description (Wiktor 1983) and hardly recognizable on the genital drawings (Wiktor 1983, 2000). During courtship, it is protruded together with an everted finger-like penial appendix assumed to be homologous to the penial gland (Wiktor 2000), and the partners are described as stroking each other with this structure rather than with the sarcobelum (Wiktor 2000, fig. 36). This would be the only known case in *Deroceras* where a penial appending structure is already everted during courtship and used for stroking. The calcareous plate of *Liolytopelte* seems not to be used during courtship except that partners of *D. caucasicum* lick the mucus off it, perhaps to prepare it for sperm exchange (Rymzhanov and Schileyko 1991; see copulation section). However, this behavior was not mentioned for *D. bureschi*, the only other species of *Liolytopelte* whose mating behavior has been observed (Wiktor 1983, 2000).

The sarcobelum of *Deroceras turcicum* is very polymorphic, varying from conical to a flat tongue, and from short to rather long (Reise and Hutchinson 2001a). The observations of mating (Gerhardt 1935, H. Reise, unpubl. obs.) indicate much similarity to *D. reticulatum*. However, it would be interesting to test whether stroking intensity and efficiency vary intraspecifically with the shape and size of this organ.

Slugs also differ in how close they get during courtship and whether there is an aggressive component in the behavior. While some species (particularly the ones with a large, flat sarcobelum) are very close from the beginning and show no, or hardly any, aggression (*e.g.*, *Deroceras rodnae* and *D. praecox*), other species keep very distant (*e.g.*, *D. gorgonium*, see above), and some regularly exchange biting attacks during the early courtship phase. Occasional biting has been observed in *D. reticulatum* (Webb 1961; Karlin and Bacon [1961] mentioned "pugnacious" strikes with the head, probably for slime feeding), *D. agreste* (Gerhardt 1933), and *D. gorgonium* (Reise *et al.* 2007).

In the particularly aggressive species *Deroceras panormitanum*, the aggression prevents them from getting close until the later stages of courtship. In this species partners initially exchange vigorous bites whenever they get closer or one tries to touch the other with its sarcobelum. Strikes onto the flank, tail, sarcobelum or head are often recognizable as bites rather than mere "licking", and the partner usually reacts with a short backward movement, frequently followed by an attack in response. The movements of the stretched sarcobela look like fencing matches in which the partners try to stroke but not to be stroked. However, bites and strokes often do not hit the partner, and many bouts of such aggression look like ritualized duels. As they do during trail following in this species, the tails seem to play an important role in the early phase of courtship: slightly lifted up from the ground and still enlarged, they are often waved just in front of the partner's face, possibly distracting the partner's biting attacks from more sensitive genital and head regions. There is strong tail lashing during phases of mutual attacks and particularly when being bitten. However, after a while, the partners slowly become less aggressive, their separation decreases, and the sarcobela finally stroke as intensely as in other species such as *D. reticulatum* (H. Reise, unpubl. obs.). *Deroceras planarioides* also exhibits much aggression and tail lashing (Gerhardt 1939).

Just before the start of copulation, the sarcobela are slightly contracted and point more or less upwards (Simroth 1885, Gerhardt 1933, Wiktor 1960). The genital openings and the bases of the sarcobela are pressed against those of the partner (Gerhardt 1933, 1936, 1939, Nicholas 1984, Rymzhanov 1994, Reise *et al.* 2007). Mouth and tentacles may "fumble" around the genital pore (*e.g.*, Gerhardt 1939, Webb 1961, H. Reise, unpubl. obs.). The anterior parts of the bodies swell, lie slightly over onto their left, and the mantles are pulled backwards. Although circling stops, the partners may entwine the anterior parts of their bodies in an even tighter position just before penis eversion. Usually, particularly when one partner shows some apparent reluctance, the slugs take up this position repeatedly, but the "reluctant" partner will always loosen the contact again before mutual penis eversion finally begins (Nicholas 1984, Reise *et al.* 2007). This is probably what Castillejo *et al.* (1989) observed in *Deroceras nitidum* and interpreted as two different, alternating kinds of stimulation: sarcobela opposite one another (the figures imply that tentacle and sarcobelum touch areas near the genital pore) alternating with stroking the partner's flank.

Kosińska (1980) reports a short transitional stage in *Deroceras sturanyi*: the sarcobelum and the "remaining parts of the copulatory organs" are everted and retracted before copulation starts. There must be some intraspecific variability, because we observed this only in one out of six matings (H. Reise and C. Natusch, unpubl. obs.). Short, partial penis eversion preceding copulation was also observed in *D. reticulatum* (Nicholas 1984).

During courtship, the ejaculate is assembled within the penis. In *Deroceras reticulatum*, sperm starts flowing from its storage site (the ductus hermaphroditicus) 10 minutes after the start of courtship, and it first appears in the penis after 20 minutes. Ten minutes later, all sperm has arrived in the penis, and within a further 10 minutes the prostate secretion has completed the sperm package (Nicholas 1984). In *D. panormitanum*, sperm was not found in the penis 10 minutes after the start of courtship, but there was an ejaculate in a specimen killed after 30 minutes (Benke 2006). Nothing is known about other species. However, it would be particularly interesting to compare these data with those from species with very short or very long courtships.

COPULATION

Copulation is the phase of sperm exchange and lasts from the start of penis eversion to the moment when the genitalia lose contact with the partner. The two everted penes swell to several times their normal size (Nicholas 1984). They appear as a bulbous, bluish transparent mass lying between the partners, and it is hard to distinguish component parts.

The speed of penis eversion and the overall duration of copulation vary considerably. Most common is a sudden, sometimes explosive, eversion and a very short copulation compared to the duration of courtship. Some species, such as *Deroceras gorgonium* and *D. turcicum*, reach maximum eversion within one second (Reise *et al.* 2007, H. Reise, unpubl. obs.). The high speed requires video-recording to discriminate the sequence of events (although video may sometimes not suffice because of the difficulty in distinguishing the parts of the penis; rapid killing of couples at different stages of copulation is thus also helpful). For this reason, most published descriptions give only an overall duration of copulation, and it is often not clear whether this includes a part of or the entire withdrawal.

As they evert, the penes entwine, in some species more tightly than in others, and they press against each other in an apparently species-specific way. The entwinement is achieved by a sickle-like curve of the everted penial bags. The shape of the penis must matter considerably for a successful "embrace" and sperm exchange. The proximal ends of some penes may even be spiral (*e.g.*, Figs. 2K-L), which might facilitate close entwinement. The extreme is *Deroceras helicoidale* Rähle, 1998 with its spectacularly prolonged, helically coiled, penial bag (fig. 2G, Wiktor 2000), but nothing is known of its copulation. In this and many other species, the curve of the penis is evident even when retracted, but in other species the curve is generated only on eversion as a result of the insertion site of the penial retractor muscle (Simroth 1885, Gerhardt 1939, Wiktor 1960). Rymzhanov (1994) stresses that Kazakh *D. laeve* differs from other *Deroceras* in that the penes are pressed less intensely against each other; he does not mention any entwinement.

There is no spermatophore, and the ejaculate is transfered as an amorphous soft mass. Simroth (1885) described the ejaculate found in the penis of *Deroceras reticulatum* before sperm exchange as fine strings rolled up into roundish bodies and surrounded by a mucous layer. At least in this species, the sperm are indeed packed in several discrete bundles and wrapped by several layers of secretions produced by the prostate (Nicholas 1984; her thesis also details secretion activities of other parts of the genital tract at successive mating stages). Simroth's assumption (1885) that the sperm mass of *Deroceras* is a precursor of spermatophores is incompatible with current knowledge of the phylogeny (Hausdorf 1998). Rather, it is probably an adaptation to a copulatory system with external sperm exchange (Nicholas 1984).

At copulation, the ejaculate is everted together with the donor's penis and transferred onto the surface of the receiver's everted penis; both partners donate and receive simultaneously. The retraction of the penis then takes the ejaculate with it (Webb 1961, 1965, Nicholas 1984, Reise 1995, unpubl. obs., Wiktor 2000, Benke 2006). In the few species for which details of sperm exchange are known, it happens at the peak of penis eversion and thus very early in copulation; this is irrespective of how long the entire copulation lasts (Reise 1995, 1997 [*Deroceras praecox*, *D. rodnae*, *D. fatrense*], Benke 2006 [*D. panormitanum*], Reise *et al.* 2007 [*D. gorgonium*]). Even in *D. sturanyi*, a species with an extremely long copulation phase, sperm exchange happens early in copulation, although the transfer is slightly slower in this species (Gerhardt 1936). It remains to be investigated what happens during the rest of the copulation in such species with long copulations. Spasms on the body surface of *D. sturanyi* (Kosińska 1980) indicate activities of internal organs. In other taxa, copulations continuing after sperm exchange have been supposed to represent efforts of donors to prevent sperm digestion and thus help the sperm reach the sperm storage site (Michiels 1998).

The ejaculate seems to be transferred from a particular area of the donor's penial wall onto a particular area of the recipient's penial wall. In *Deroceras gorgonium*, the ejaculate is "slapped" onto the partner's penis (onto an area on the wall just distal to the base of the sarcobelum), and the donating part of the penis slackens immediately after this. In *D. reticulatum*, the ejaculate is collected at the base of the penial gland in the donor's penis and transferred onto the folds in the proximal part of the receiver's penis (Webb 1961, Nicholas 1984).

The majority of *Deroceras* species have one or more glandular penial side pockets (Sirgel 1973, Wiktor 2000). At least in *D. praecox*, *D. panormitanum*, and *D. gorgonium* these are everted during copulation (Webb 1961, 1965, Benke 2006, Reise *et al.* 2007, H. Reise, unpubl. obs.). Their functions are largely unknown, but, at least in some species, one pocket seems to serve as an ejaculate-holding bag prior to transfer, probably to ensure a more exact positioning of the ejaculate onto the partner's penis. The function of the additional bags occuring in a number of species is unknown. Most is known about *D. panormitanum*. The penis of this species has two side pockets: the longer one ("terminal lobe" of Webb 1961, 1965, "longer penial diverticle" of Sirgel 1973) is everted at the beginning of copulation and slackens immediately after maximum eversion; the shorter one ("medial lobe" of Webb 1961, 1965, "shorter penial diverticle" of Sirgel 1973) is everted slightly later (Benke 2006), or considerably later (Webb 1961), pressed onto the partner's shorter pocket and remains inflated for a longer time, during which pumping movements of the penial mass can be observed (Benke 2006). The longer pocket is filled with the sperm mass during courtship (Sirgel 1973, Benke 2006) and it also adds its own, probably holocrine, secretions (Sirgel 1973). At eversion, the curved longer pocket is laid around the base of the receiver's sarcobelum, to which the ejaculate is then attached (Benke 2006). Little or no sperm can be found in the shorter side pocket (Sirgel 1973, Benke 2006). This has a different internal structure from the longer pocket and produces two different types of secretions (Sirgel 1973). Sirgel (1973) assumes that these secretions are added to the ejaculate during the eversion process, but while it is still in the lumen of the penis. In that case, it is be puzzling why this shorter pocket is everted later than the other one and remains inflated for so long. However, this secretion must be the reason why Webb (1961) thought that the ejaculate is transferred with the second, shorter pocket. The latter was also reported by Barker (1999) but the perfect agreement between these publications suggests that Barker based his account on Webb's.

During courtship of *Deroceras caucasicum*, the sperm mass is put into, and wrapped by, an albumen membrane extended like a hammock within the donor's penis between two nodular appendices. This package is then laid onto the receiver's calcareous plate during copulation (Rymzhanov and Schileyko 1991). This is apparently the function of the plate, whose presence defines the subgenus *Liolytopelte*.

In *Deroceras panormitanum*, *D. caucasicum*, *D. reticulatum*, *D. sturanyi*, and Kazakh *D. laeve*, the ejaculate is transported through the vas deferens into the penis before copulation starts (Simroth 1885, Wiktor 1960, Webb 1961, Nicholas 1984, Rymzhanov and Schileyko 1991, Rymzhanov 1994, Benke 2006). However, Gerhardt (1933) and Reise (1995) reported seeing the ejaculate being expelled through the vas deferens during copulation of *D. agreste* and *D. rodnae* respectively. Gerhardt (1939) reported this also for *D. panormitanum*, which is in conflict with other, more thorough, studies of this species based on rapid killing at different mating stages (Webb 1961, 1965, Benke 2006). This calls for a critical reexamination in *D. agreste* and *D. rodnae* using the rapid-killing technique.

The ejaculate is visible either during the entire process of sperm exchange or at least when the slugs separate. In at least some species, the ejaculate forms a longish package, sometimes stretched so much that one end still sticks out of the genital orifice when the penis is fully retracted. The color of ejaculates varies between white and yellow (Gerhardt 1933, 1936, 1939, Wiktor 1960, H. Reise, unpubl. obs.); it is white in *Deroceras praecox* but yellow in its sibling species *D. rodnae* (Reise 1995).

Species that have an appending penial gland (at the proximal end of the penis, Fig. 1) evert it during copulation (Simroth 1885, Gerhardt 1933, 1939, Webb 1961, 1965, Nicholas 1984, Castillejo *et al.* 1989, Reise 1995, Wiktor 2000, Reise *et al.* 2007. Runham (1978) wrongly wrote that it is everted during courtship in *Deroceras reticulatum*, and Wiktor (1983, 2000) suggested this for *D. bureschi*—see section on courtship). In species in which the gland is sufficiently large, it usually spreads over the partner's body (Fig. 6; Webb 1961, 1965, Nicholas 1984, Castillejo *et al.* 1989, Reise and Hutchinson 2001b, Benke *et al.* 2005, Benke 2006, H. Reise, unpubl. obs.). This eversion is striking in species with a large gland (*e.g.*, *D. panormitanum*), which can span most of the body length. The gland is always everted for only a short time and retraction starts immediately after full eversion (Webb 1961, Reise 1995, unpubl. obs., Benke 2006). However, the timing varies between species. In some species with a very sudden and quick copulation, the partners evert their glands more or less simultaneously along with the full penis eversion and sperm exchange (Reise 1995, unpubl. obs.). However, in *D. gorgonium*, where copulation is also very quick, video recording showed that the highly branched glands are everted immediately after the ejaculate has been transferred onto the receiver's penis (Reise *et al.* 2007). Similarly, Wiktor (1960) and Webb (1961) observed that in *D. reticulatum* the gland is everted at the end of copulation when other parts of the penis are collapsing. In *D. panormitanum*, the comparatively slow copulation makes it even clearer that gland eversion is after sperm exchange, when the other parts of the penis have begun to retract (Fig. 5; Webb 1961, Reise and Hutchinson 2001b, Benke *et al.* 2005, Benke 2006). Moreover, the second partner begins to evert its gland only when the first partner has finished, or almost finished, retracting its penis and gland and often has already started to crawl away. Particularly in the latter case, some or all fingers of the gland may miss their target and spread on the ground (H. Reise, unpubl. obs.). Benke (2006) observed intervals of 10-72 s (mean = 32 s) between the eversions of each partner's gland, but she did not find a clear difference in the

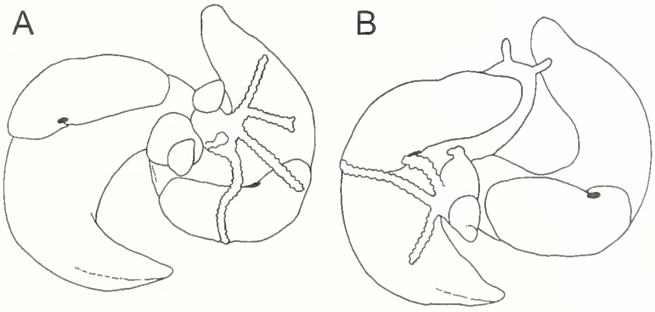

Figure 6. Eversion of the penial gland in *Deroceras panormitanum*. A, Maximum eversion of the left slug's gland. B, Eversion of the right slug's gland; the partner has already retracted and is crawling away. Drawn from two video frames.

success of first and second gland eversions: out of 35 matings, Benke observed full or partial misses for the first eversion in four cases and for the second in three cases.

The only report that the penial gland of one partner does not evert during a copulation is from two matings (out of 27) of *Deroceras panormitanum* (Benke 2006, personal communication). The penial gland of one of these slugs was still filled 120 min after copulation, indicating that the secretion had been produced but not transfered. The penial gland of the other slug, killed after 30 min, was empty; it might not have produced a secretion or eversion might have been overlooked. It can be difficult to spot the eversion if the gland spreads underneath the partner's body, as appears to be common in *D. gorgonium* (Reise *et al.* 2007). Nothing is known about how species with a very small penial gland, such as *D. agreste*, use it.

The function of the penial gland is not known. Webb (1961) assumed that it functions for "semen-securing and retaining" during retraction, but there is no evidence for this (Nicholas 1984, Benke 2006, H. Reise, unpubl. obs.). However, the observation that it is everted only after sperm exchange in *Deroceras panormitanum* has prompted the hypothesis that it transfers a secretion to manipulate the partner physiologically so as to increase the chance of paternity (Reise and Hutchinson 2001b; *cf.* the proven function of the dart in *Cornu aspersum* [Müller, 1774]; Koene and Chase 1998, Rogers and Chase 2001, 2002, Landolfa *et al.* 2001, see also the discussion section). The secretion would thus act as an allohormone (Koene and Ter Maat 2001). Histological studies and direct observations have shown that the penial gland is filled up during early courtship and that this secretion is transferred onto the partner's skin (Nicholas 1984, Benke et al. 2005, Benke 2006). Observations that mating partners in *D. panormitanum* try to lick off this secretion from their own body just after copulation (Benke 2006) might be efforts to escape manipulation by the partner. At least some of the secretion accumulating in the penial gland before copulation is transferred from the prostate (Nicholas 1984), but there are also indications of aprocrine secretion in the penial gland itself (Sirgel 1973, Nicholas 1984, Benke 2006).

Although the usual mode in *Deroceras* is simultaneous mutual sperm exchange, unilateral sperm transfer occurs occasionally. In *D. rodnae*, Reise (1995) observed a few such cases associated with unusual behavior of the sperm donor. The donor slug tried to keep contact with the recipient by following, with genitalia still partly everted, but the recipient fully retracted its genitalia and showed no further interest. I. Schulze (pers. comm.) did not find an ejaculate in 6 out of 105 individuals of *D. panormitanum* killed immediately after copulation. Benke (2006) killed couples of the same species 10–90 min after copulation and found that in 9 out of 39 individuals there was no ejaculate in the penial sac (where the received sperm mass should have been). At least 4 of the partners of these 9 slugs had one sperm package in their penial sac as well as another one in the long side pocket; that is, one ejaculate that they had just received and one prepared for donation. Gerhardt (1933) reported ejaculates of *D. reticulatum* which missed the receiver and ended up on the ground (I. Schulze [pers. comm.] observed another instance). Such lost ejaculates, or partial ejaculates, are also implied by Nicholas' (1984) statement that after copulation *D. reticulatum* eat remaining sperm along with accumulated mucus. Cases of unilateral sperm exchange where an ejaculate was produced but not donated successfully (either kept by the sperm owner or lost during transfer) might represent accidents. Alternatitively, they might be caused by one partner deliberately not performing one of the two sexual roles. Cheating on the reciprocal sperm exchange by avoiding donating sperm is an unlikely explanation, because this partner has paid much of the cost of the male role by having produced an ejaculate. However, deliberate sperm rejection by a receiver would be more plausible. Sperm rejection (or eating) might be advantageous over sperm digestion in the bursa copulatrix if the ejaculate contains manipulating substances causing costs to the receiver.

Kosińska (1980) observed several couples of *Deroceras sturanyi* in which only one partner fully everted the penis, and she concluded that only one partner received sperm. However, I doubt that any sperm transfer took place because this must surely require not only a donating but also a receiving penis. This is also relevant to the question of whether aphallic indviduals of *D. laeve* can receive sperm from an euphallic individual (see sections on genital morphology and withdrawal). The only other recorded cases of unilateral penis eversion are from mixed couples of two different species, *D. praecox* and *D. rodnae* (Reise 1995; see section on timing).

Webb (1961) noted that copulating *Deroceras reticulatum* "invariably" take up their own ejaculate or a mixture of both partners' ejaculates, but he did not indicate what evidence prompted this conclusion.

The duration of the copulation phase varies remarkably between species (Table 2). Gerhardt (1939) classified *Deroceras* into two groups: (1) species with short copulations during which partners entwine further: *D. reticulatum*, *D. agreste*, *D. turcicum*, and *D. planarioides*; (2) species with long copulations during which partners do not entwine further: *D. panormitanum*, *D. lombricoides*, and *D. sturanyi* (which he called *D. laeve*, see introduction; mistakenly, on p. 199, he listed *D. reticulatum* and *D. agreste* instead). *Deroceras fatrense*, *D. gorgonium*, *D. praecox*, *D. rodnae*, and Kazakh *D. laeve* should now be added to the fast group. Gerhardt's (1939) hypothesis of a consistent association between speed of copulation and further entwining during copulation must now be rejected: *D. praecox*, a species with a very fast copulation, does not entwine further. Instead, the partners are even pushed apart by the everting penial mass (H. Reise, unpubl. obs.). I observed the same phenomenon in video-recorded copulations of *D. turcicum* (H. Reise, unpubl. obs.), which is in disagreement with Gerhardt (1935). However, he might have worked with a different species (he called his slugs "*D.* aff. *turcicum*").

The species with "fast" copulations form a rather homogeneous group with a copulation time of less than one minute. In contrast, the "long" copulations of the remaining species vary from three minutes to several hours and their intraspecific variability is much higher: about 3-12 min in *D. panormitanum* and 60-148 min in *D. sturanyi* (Table 2). Examination of additional species may well establish more of a continuum between the groups with short and long copulations, but even then the variation in duration would remain to be explained.

WITHDRAWAL

Irrespective of whether the copulation is short or long, penis retraction is usually a rather fast and straightforward process once started (Gerhardt 1935, 1936, 1939, Reise 1995, unpubl. obs.) although it is much slower than the eversion. There are a few exceptions: (i) delayed or only partial retraction by one of the partners in rare cases of unilateral sperm exchange (see preceding section); (ii) late eversion of the penial gland in *Deroceras panormitanum* when the main part of the penis is already retracting (see preceding section); (iii) one partner remaining inactive at the mating site with fully everted penis in *D. sturanyi* (sometimes) and Kazakh *D. laeve* (always), with or without apophallation (see below). Because eversion of the penial gland probably plays an important role and overlaps other components of copulation, I include gland eversion as part of the copulation phase and define the end of the copulation phase (and the start of the withdrawal phase) as when the genitalia no longer have any contact with the mating partner. As a consequence, penis withdrawal and sperm uptake may start before the withdrawal phase.

The most common mode seems to be that both partners withdraw more or less simultaneously, performing intense pumping and rocking movements with the anterior body. As soon as the genitals untangle, the slugs separate and may crawl away from each other before finishing withdrawal. Usually the partners show no further interest in each other, with the exceptions (i) and (iii) mentioned in the preceding paragraph. The sarcobelum is the last part to disappear inside. Some ejaculate may stick to the last genital parts to remain everted, and then the slug may turn its head to its genital orifice and apparently push the sperm mass in (Reise 1995). However, Benke (2006) observed two cases in *Deroceras panormitanum* where one partner tried to eat ejaculate sticking to its own, not yet fully retracted, genitalia. So the question of whether the slugs are assisting uptake of the ejaculate or eating it (*cf.* Karlsson and Haase 2002) needs reinvestigation. Eating could also be a last resort if the uptake is going to fail, similar to when the ejaculate has been lost onto the ground.

Some unusual behaviors during the withdrawal phases have been reported in *Deroceras sturanyi* and Kazakh *D. laeve*. Probably in many matings of *D. sturanyi*, only one partner retracts its penis immediately after copulation and crawls away. The other partner remains motionless at the mating site for some time with its penis still everted and slowly retracts it later. Kosińska (1980) apparently observed this in all matings but unfortunately does not tell how many she observed or how much later the second partner retracted. We found a very high variability in the mating behavior of *D. sturanyi* collected in Germany, only about 200 km away from Kosińska's population. In only one out of six full matings did one partner retract its penis considerably later (70 min) than the other. In four cases, both partners retracted more or less simultaneously, with not more than one minute difference. In one pair, one partner left only the sarcobelum everted, which was retracted after 25 min (H. Reise and C. Natusch unpubl. obs.). Rymzhanov (1994) and Gerhardt (1936) did not observe delayed retraction in any of the matings of 18 *D. sturanyi* from Kazakhstan or three matings of *D. sturanyi* from Germany.

Rymzhanov (1994) has reported retraction by only one partner as a regular pattern in matings of 12 Kazach *Deroceras laeve*. Moreover, the penis of the slug remaining at the mating site was not retracted later but bitten off by its owner and finally eaten by the other slug, which returned to the mating site and assisted amputation by pulling. Apophallation had previously been reported only from *Ariolimax* (Mörch, 1860), in which the penis may also be eaten by the partner (Leonard *et al.* 2002). These slugs are from a different family, Arionidae. They copulate by mutual or unilateral penis intromission and one penis (or both) is occasionally bitten off, usually after a period of struggle and pulling by the owner (implying that the penis gets trapped in the partner's genital tract (Mead 1943, Harper 1988, Heath in Pilsbry 1948: 710-711). Rymzhanov's (1994) observation of Kazach *D. laeve* is even more remarkable because the penis is not trapped in the partner's genital tract, and because apophallation is the rule rather than the exception. It seems hard to imagine a reason why an individual should voluntarily initiate amputation of its own penis; if it were the metabolic cost of keeping the organ when it will not be used again, it is puzzling why the slug does not eat it itself. One possible explanation might be that the amputee has been manipulated by the partner by transfer of a secretion which inhibits retraction ability. The partner might be interested not only in gaining an additional food source but also in restraining it from remating and thus preventing sperm competition and/or shifting sex allocation towards the female function (the external mode of sperm exchange in *Deroceras* makes it improbable that individuals without a penis can donate or even receive sperm, in contrast with *Ariolimax* where aphallate individuals can still receive sperm because there is intromission [Leonard *et al.* 2002]). However, this explanation would only be plausible if the amputee has been able to take up the received ejaculate without penis retraction. Rymzhanov (1994) reported that the amputee was always the follower during precourtship trail following, so its fate was fixed at the start of mating. His paper further suggests that this apophallation is the origin of aphallic and hemiphallic individuals in *D. laeve* (see section on genital morphology). However, in German and North American populations of *D. laeve*, individuals that have grown up in isolation are often aphallic (Barth 2001, V. Barth and H. Reise, unpubl. obs., Reise and Hutchinson 2002, Jordaens *et al.* 2006). The phenomenon and the species identity are worthy of further investigation.

After full withdrawal, there is usually some eating of mucus by one or both partners. They may return to the mating site and lick the mucus-covered ground (Kosińska 1980, Nicholas 1984, Rymzhanov 1994, H. Reise, unpubl. obs.) or lick their own body surface (Benke 2006, H. Reise, unpubl. obs.), but there is much intraspecific variation. The function of this behavior is unknown. The mucus might simply serve as a nutritious substance. But a slug licking its own body might be trying to consume, and thus inactivate, an allohormone transferred by the partner by its sarcobelum during courtship or by its penial gland during copulation (see those sections).

Full withdrawal of all genital parts into their original position takes much longer than the withdrawal phase. The retraction of the penial gland of *Deroceras reticulatum* takes 75 minutes according to Nicholas (1984) and "several hours" according to Webb (1961), but it might well take more time in species with more highly branched or longer penial glands.

There is some controversy about the fate of the sperm after penis retraction. Because the sperm is transfered during copulation onto the penis, immediately after retraction it must lie somewhere in the main penial bag (Nicholas 1984, Benke 2006, Reise *et al.* 2007. It must then move via the atrium towards the bursa copulatrix and oviduct. However, there is an array of opinions, some better supported than others, about how long the ejaculate remains in the penis, whether the sperm has to enter the bursa copulatrix or not before proceeding to a sperm storage site, and whether it migrates along the female or male groove of the spermoviduct (Webb 1961 Sirgel 1973, Nicholas 1984, Tompa 1984, Rymzhanov and Schileyko 1991, Wiktor 2000).

DISCUSSION

Despite the mixed quality of observations and the small proportion of species examined, considerable variation in the mating behavior of *Deroceras* is already apparent. However, the extent to which this variation is intraspecific or interspecific is not always clear, and there is a need to investigate the intraspecific variation between and within populations. The genus contains many nominate species of similar morphology; mating behavior can sometimes provide a suite of extra characters to help resolve such taxonomic problems. Because mating behavior is a potential isolating mechanism, it will also be interesting to study mating behavior across the contact zones of closely related species with parapatric distributions.

Mating in *Deroceras* involves very complex copulatory organs and behavioral patterns. After a comparatively long time of caressing with the sarcobela, copulation usually begins very suddenly and sperm transfer occurs often, or maybe always, at the very beginning of copulation. This demands perfect coordination and penis alignment between the partners. It seems plausible that slight discrepancies in the preceding courtship phase (*e.g.*, due to different shape and movement of the sarcobelum) or during sperm transfer (*e.g.*, due to shape differences of the penes and thus less perfect entwining) can impair sperm exchange. I have often observed difficulties between conspecific mating partners from different populations, which might be caused by such slight differences (Reise 2001, unpubl. obs.). In other taxa, intraspecific variation of sexual behavior among populations is common, and morphological, behavioral, and other traits that determine mate recognition (*e.g.*, pheromones) may evolve quickly and play a significant role in allopatric speciation (Arnquist and Danielsson 1999, Verell 1999). If, as hypothesized in the introduction, sexual conflict between mating partners drives rapid evolution of penial morphology and mating behavior in *Deroceras*, interpopulation incompatibilities, rapid speciation, and many species might be the consequence.

There is so far little evidence addressing the importance of sexual conflict in *Deroceras*. The unusual behavior of sperm donors in rare cases of unilateral sperm transfer in *D. rodnae* (see section on copulation) might be interpreted as a preference for the female (sperm receiving) role and for occasional cheating in a mating system based on reciprocity (according to the hermaphrodite's dilemma model: Leonard

1990, 1991, 2005). Both the preference and the cheating suggest partner conflict over sexual roles. However, the cases of unilateral sperm transfer when both ejaculates had been produced might represent sperm rejection, *i.e.* preference of the male role. The preference for one of the two sexual roles has been repeatedly predicted for mating systems of simultaneous hermaphrodites, but it is controversial which factors should decide the prefered role (reviewed by Anthes *et al.* [2006]).

Michiels (1998) has suggested that a preference for the female role could result in elaborate behavior to stimulate the partner into donating sperm and to assess its readiness to do so; until reciprocity is assured, no sperm should be donated. Although we know almost nothing of the mechanisms of stimulation or assessment, this might explain the very long courtships in *Deroceras* and some species of *Limax* Linnaeus, 1758 (Gerhardt 1933), which surely entail higher costs than short courtships (Baur 1998). Assurance of reciprocity and/or manipulation of the mating partner into accepting sperm have been proposed as an explanation of elaborate courtship in a nudibranch (Karlsson and Haase 2002). A further possibility is that the length and vigor of courtship have been sexually selected as honest signals of the partner's condition and thus of its genetic quality. Although complete mate rejection seems to be rare once courtship has started, "weak" courtship behavior might make it more likely that sperm exchange is unilateral, or might reduce how much sperm is donated or the partner's use of donated sperm.

The length of courtship contrasts with the extremely fast penis eversion and sperm transfer in some species of *Deroceras*. Tight physical contact of penes during copulation might ensure reciprocal sperm exchange (*cf.* the entwined penes in *Limax*; Michiels 1998). However, a possible consequence of the rapidity of copulation is that once an individual has everted its sperm mass with its penis, it may have no chance to withhold its ejaculate, should it realize that the partner is not going to donate. This conflicts with Davison *et al.*'s (2005) assumption that in systems with simultaneous reciprocal mating, cheating is possible only after intromission (meaning sperm transfer). The ability to cheat before sperm exchange might be a peculiarity of groups with external penis-to-penis sperm transfer. It remains unclear what has favored the evolution of the rapid penis eversion and sperm transfer; the advantage of being slightly quicker to evert than the partner seems unlikely to be in unilaterally snatching the partner's ejaculate, because the penis has to be fully everted before the ejaculate becomes available. It seems more compatible with a preference for the sperm-donating role.

Another possible cause of partner conflict, widely assumed to exist in gonochorists as well as hermaphrodites, concerns control over fertilization. Sperm donors should endeavor to ensure that their sperm fertilizes the partner's eggs. If the manipulations to achieve this, or the loss of control itself, involve a cost to the sperm receiver, counter-adaptations are expected. In *Deroceras* the most promising evidence of a manipulation comes from the appending penial gland in species in which it is everted only after sperm exchange. This cannot serve sperm exchange but might manipulate the partner into using the donor's sperm, as do allohormones transferred by the love dart of *Cornu aspersum* (see section on copulation). However, other functions are also possible, such as increasing the number of eggs available for fertilization as might be the case in *Lymnaea stagnalis* (Linnaeus, 1758) (Koene *et al.* 2006) or delaying subsequent mating by an antiaphrodisiac effect (*cf.* Andersson *et al.* 2004), or marking the partner so as to prevent repeated mating with the same mate (*cf.* Ivy *et al.* 2005).

The unusual external mode of sperm exchange could also indicate sexual conflict over control of egg fertilization: Emberton (1994) hypothesized that the extremely prolonged intertwining of penes of some polygyrid and *Limax* species represent male efforts to place the ejaculate as far away from the partner's gametolytic bursa copulatrix as possible. The evolution from penis intromission into the bursa trunk towards external transfer from penis to penis has evolved at least four times independently within the pulmonates (Emberton 1994). However, Solem (1974) thought that elaborate genital structures, including very prolonged penes, evolved to enhance species recognition—that is, as an isolation mechanism. I tend to agree with Emberton (1994) and think that circumvention of female control may have driven the abandonment of direct sperm transfer into the bursa copulatrix. However, I do not agree that a longer penis would be even better in circumventing the bursa copulatrix than a moderately long one. Allosperm is believed to travel up the spermoviduct, and thus to pass the entrance of the bursa trunk, irrespective of penis length. Another possible explanation for the very prolonged penes of polygyrids and *Limax* is that they have been sexually selected as condition-dependent cues used to assess the desirability of the mating partner. In contrast, most of the various extravagant penial structures in some *Deroceras* species (Fig. 2) seem unlikely to be reliably condition-dependent (their size is still rather trivial compared with that of the body), but they remain most explicable by some form of arms race.

There are many other aspects of mating behavior and morphology that one might examine to discover indications of partner conflicts in *Deroceras*: the role of the (probably secretion-transferring) sarcobelum, the function of additional penial side pockets (besides ejaculate-holding), the processes occuring after sperm exchange before penis withdrawal, the occasionally long-delayed penis withdrawal in *D.*

sturanyi, and apophallation in Kazakh *D. laeve*. Other suggestions for future research are the study of mating in species that represent morphological or behavioral extremes, and the relating of behavior to morphology in species polymorphic for a penial structure (such as the highly variable sarcobelum in *D. turcicum*: Reise and Hutchinson 2001a). Several other fruitful lines of research depend on the development of a molecular-based phylogeny.

Finally, I will make the following recommendations for studies of mating behavior in *Deroceras*. (i) Observations should include all mating phases, which normally requires introducing individuals to each other in the laboratory. (ii) Records should be kept of matings that cease before copulation, in particular noting at what stage this happens and the size and mating histories of the individuals involved. (iii) Individuals should be followed so as to reveal correlations in which partner takes which role at different stages. (iv) Note whether both partners donate and receive an ejaculate, and, if not, whether both partners have manufactured sperm packages. (v) Direct observations should be complemented by video-recording and rapid killing of couples at successive mating stages. (vi) The studies should include several pairs and, preferably, more than one population.

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Reproductive biology and mating conflict in the simultaneously hermaphroditic land snail *Arianta arbustorum**

Bruno Baur

Department of Environmental Sciences, Section of Conservation Biology, University of Basel, St. Johanns-Vorstadt 10,
CH–4056 Basel, Switzerland, Bruno.Baur@unibas.ch

Abstract: This review summarizes the present knowledge on the reproductive biology, mating system, sperm competition, sex allocation, and mating conflict in the simultaneously hermaphroditic land snail *Arianta arbustorum* (Linnaeus, 1758) (Helicidae). Field studies and controlled laboratory experiments indicate that mating is random with respect to shell size. However, subtle effects of inbreeding (reduced hatching success of eggs and viability of juveniles) and outbreeding were found. Individuals of *A. arbustorum* mate repeatedly with different partners and store viable sperm for more than one year. Spermatophore transfer is highly reciprocal, but the number of sperm they contain (800,000-4,000,000) is not necessarily equal. Snails need 3-4 weeks to replenish their autosperm reserves after a successful copulation. Sperm are monomorphic. However, there is considerable among-population—and to a minor extent—within-population variation in total sperm length. Sperm utilization patterns in double-mated individuals of *A. arbustorum* revealed striking differences among individuals. There is a huge variation in the structure of the spermatheca, which consists of 2-9 blind tubules. Different lines of evidence suggest that the snails might be able to store and expel sperm stored in single tubules and thus promote a selective fertilization of eggs (cryptic female choice). Maternal investment in eggs is considerable. Snails mated 1, 2, or 3 times showed that irrespective of the number of matings the individuals devoted >95% of the resources into the female function.

Key words: Gastropoda, mating system, reproductive behavior, sex allocation, sperm competition

Traditional models of sexual selection explain the evolution of secondary sexual traits (mainly in males) and preferences for reproductive partners displaying such traits (mainly in females; Andersson 1994). Although these models differ in how sexual selection operates, they all propose that partner choice increases both average male and average female fitness in a population (Pizzari and Snook 2003). Recent theoretical and empirical work, however, has stressed that sexual conflict may be a potential broker of sexual selection. When the fitness interests of females and males diverge, a reproductive strategy that increases the fitness of one sex may decrease the fitness of the other sex. In this way unequal reproductive investments per gamete (egg versus sperm) lead to sexual conflicts (Trivers 1972). In recent years, experimental evidence for sexual conflicts has been reported in a variety of gonochoristic animals (Chapman *et al.* 2003, Arnqvist and Rowe 2005). Mechanisms of sexual conflicts are of particular interest in simultaneous hermaphrodites (Michiels 1998).

In hermaphrodites, selection for female traits cannot be independent from selection for male traits of the same individual. Sexual selection for traits related to mate attraction is assumed to be weaker in hermaphrodites (Greeff and Michiels 1999a), but because many simultaneous hermaphrodites mate repeatedly and store sperm for long periods, they are affected by forces similar to those leading to complicated mating strategies and sperm competition in animals with separate sexes (Charnov 1996, Michiels 1998, Angeloni *et al.* 2003). However, unlike gonochoristic species, simultaneous hermaphrodites have an additional reproductive strategy; they can adjust the ratio of resources invested into reproduction in the female role versus the male role, depending on current selection pressures and environmental conditions (Charnov 1982). Sex allocation complicates sexually selected strategies because any increased investment in one sexual role results in a decreased investment in the other.

Pulmonate gastropods are excellent study organisms for determining the mechanisms and effects of sperm competition and sex-specific reproductive allocation on both the fitness of snails and the conflict between male and female function within an individual (Baur and Baur 2000). Although the theoretical framework for sexual conflicts in simultaneous hermaphrodites already exists, such conflicts have rarely been examined experimentally in terrestrial gastropods (for an exception see Chase 2007).

The aim of this review is to summarize the present knowledge on the reproductive biology, mating system, sperm competition, sex allocation, and mating conflict in the pulmonate land snail *Arianta arbustorum* (Linnaeus, 1758). The strength of this model system comes from the possibility of conducting field experiments and of studying the behavior of individual snails under controlled conditions; the ability to produce laboratory crosses with relative ease; and the availability of well-established techniques (*e.g.*, to estimate the number of sperm delivered and the number of eggs produced [Locher and Baur 1997], and to measure sperm length, sperm velocity, motility and longevity [Minoretti and Baur 2006]; two novel non-invasive techniques for isolating DNA and a set of microsatellite markers which can be used for paternity analysis [Locher and Baur 2001, Armbruster *et al.* 2005]). By compiling the existing information, this review should stimulate future studies on mating systems and reproductive behavior in simultaneously hermaphroditic gastropods.

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NATURAL HISTORY

Members of the species *Arianta arbustorum* are simultaneously hermaphroditic, helicid land snails common in moist habitats of northwestern and central Europe, living at elevations of up to 2700 m above sea level in the Alps (Kerney and Cameron 1979). Adult shell size diminishes with increasing elevation (population means of shell breadth ranging from 13 to 23 mm; Burla and Stahel 1983, Baur 1984a, Baur and Raboud 1988). The species shows a high phenotypic variability in shell and soft body color that is related to habitat and elevation (Burla and Stahel 1983, Gittenberger 1991, Gosteli 2005). Variation in shell color is continuous between large brown morphs, occurring mainly in forests in the lowlands, and small yellow morphs in alpine grasslands at high elevation. Population density is highly variable in *A. arbustorum*, ranging from 0.01 to 11 adults per m^2 (Baur 1986a, 1988a). On mountain slopes, *A. arbustorum* can be continuously distributed over large areas and thereby reach huge population sizes (Baur 1986a). In contrast, partly or completely isolated populations consisting of only a few individuals can persist in small patches of marginal habitat (Baur 1993a, Akçakaya and Baur 1996). Information on patterns of daily movement and dispersal in *A. arbustorum* can be found in Baur (1986a), A. Baur and B. Baur (1990, 1992, 1993), Baur *et al.* (1997), and Kleewein (1999).

Arianta arbustorum has determinate growth. Sexual maturity is attained after the completion of shell growth. However, matings involving individuals that had not yet finished their shell growth have been observed in a few cases (Baur 1984b). Age at maturity increases from 2 years in snails living in the lowlands to 5 years in individuals in alpine populations, but median adult longevity (3-3.5 years; maximum 14 years) and adult survival rates (0.5-0.7 per year) are approximately the same at all elevations (Baur and Raboud 1988). Individuals of *A. arbustorum* mate repeatedly during the reproductive season (Baur 1988b). In the field, snails deposit one to three egg batches, each consisting of 20-60 eggs, per reproductive season (Baur and Raboud 1988, Baur 1990a, B. Baur and A. Baur 1993). During the winter snails hibernate in leaf litter or soil (Terhivuo 1978).

Arianta arbustorum exhibits large interpopulational genetic variation (Arter 1990, Haase *et al.* 2003) and shows considerable variation in behavior and rate of parasitic infestation (Baur 1986b, 1994a, Baur and Gosteli 1986, Baur and Baur 2005). In some populations, individuals of *A. arbustorum* are infested by the mite *Riccardoella limacum* which sucks blood and lives in the snails' lungs. Prevalence of mite infection ranged from 45.8 to 77.8% in four natural populations of *A. arbustorum* while in seven other populations no infected snails were found (Baur and Baur 2005). Information on density-dependent growth and potential predators on *A. arbustorum* is summarized in Reichardt *et al.* (1985). Aspects of food choice in *A. arbustorum* have been examined by Speiser and Rowell-Rahier (1991, 1993) and Speiser *et al.* (1992).

MODE OF REPRODUCTION

Arianta arbustorum was long believed to reproduce exclusively by cross-fertilization (Lang 1904). Chen (1994) reared 44 individuals in isolation from the subadult stage and recorded their reproductive performance for 1, 2, or 3 years under laboratory conditions after they had attained sexual maturity. The snails produced eggs without being mated, however, in a significantly lower number than did mated control snails. In their first reproductive season, only 1 egg hatched out of 284 eggs (hatching success 0.4%) produced by 33 snails. In the second reproductive season, 32 of the 671 eggs laid by 29 snails hatched (hatching success 4.8%). In the third reproductive season, 23 of the 191 eggs laid by 6 snails hatched (hatching success 12.0%). The percentages of snails that laid fertile eggs were 3.0% (1 of 33 snails), 31.0% (9 of 29 snails), and 33.3% (2 of 6 snails), respectively, for the 3 years after maturation. The number of hatchlings produced by unmated snails was 1-2% of that produced by mated snails of the same age. This indicates that *A. arbustorum* is able to self-fertilize, but with a great reduction in fitness. Cross-fertilization is the dominant mode of reproduction in this species. Self-fertilization might occur only if no mating partners were available over long periods.

MATING PATTERNS

Size-assortative mating is a common pattern in natural populations of many invertebrate and vertebrate species.

Hermaphroditic land snails would greatly enhance their reproductive success by choosing large mates since female fecundity (number of clutches, clutch size, and egg size) is positively correlated with shell size (Wolda 1963, Baur 1988a, Baur and Raboud 1988). Ridley (1983) suggested that size-assortative mating patterns should occur in simultaneously hermaphroditic land snails with reciprocal fertilization and size-related female fecundity. He argued that all individuals invest substantially (all their eggs) in mating, so there will be selection for careful mate choice. In another approach, Parker (1983) proposed a model for indiscriminate mate choice (random mating). This should occur when there is little variance in mate quality in both sexes, and/or when search costs for mates are high (*e.g.*, low encounter rates due to low population densities or low mobility). Baur (1992a) examined mating patterns in natural populations of *Helix pomatia* Linnaeus, 1758 and *Arianta arbustorum*. In a large population of *H. pomatia* (700-1000 individuals) in Sweden, snails showed a slight (but non-significant) tendency towards size-assortative mating, whereas mating in a subalpine population of *A. arbustorum* in Switzerland was random with respect to size. Baur (1992a) also conducted controlled choice experiments to test whether individuals of *A. arbustorum* discriminate between mating partners of different sizes and whether a large shell size might be of advantage in groups of courting snails to increase mating success. In mate-choice tests with snails of different shell size, pairs formed randomly with respect to size. Courtship was neither hindered nor prolonged in pairs with large size differences. In the second experiment, a large *A. arbustorum* was placed close to two courting conspecifics (both smaller). The larger snail interfered with the courting snails, but in general did not displace one of them. Courtship progressed to copulation only if one of the three snails ceased to court; this was independent of the size of the individual. Thus, a large shell did not increase mating success. Time-constraints of locomotory activity and high costs of searching for a mate can explain the prevalence of random mating patterns in simultaneously hermaphroditic land snails (Baur 1992a).

Mating has also been reported to be random with respect to shell size, shell color, and banding pattern in *Cepaea nemoralis* (Linnaeus, 1758) (Schilder 1950, Schnetter 1950, Lamotte 1951, Wolda 1963). Mating was also random between resident and introduced individuals of *Helix pomatia* (Woyciechowski and Lomnicki 1977). Mate-choice tests with *Arianta arbustorum* from geographically isolated populations in Sweden and Switzerland revealed that snails from three populations preferred to mate with snails from their population of origin although no interpopulational differences in latency or duration of courtship were found (Baur and Baur 1992a). Mating preferences could partly be explained by differences in mating propensity in two of the three populations, but not in matings between a Swedish and a Swiss population. Cross-breeding demonstrated a high degree of reproductive compatibility between these two distant populations. In contrast, pairs involving individuals from two distant Swiss populations had a reduced fertility. The experimental results indicate effects of outbreeding depression between distant populations of *A. arbustorum*. However, the extent of outbreeding depression seems not to be related to the geographical distance between populations.

Mating between closely related individuals can incur substantial fitness costs (*i.e.*, inbreeding depression). For simultaneous hermaphrodites such as *Arianta arbustorum*, inbreeding depression is regarded as the most important selective force acting against self-fertilization, and maintaining outcrossing (Ghiselin 1969, Jarne and Charlesworth 1993). B. Baur and A. Baur (1997) performed mate-choice tests to examine whether individuals of *A. arbustorum* discriminate between full-sibs and non-sibs from the same population and whether incestuous matings reduce the snails' subsequent reproductive success. Full-siblings (F_1) were raised under laboratory conditions. Snails mated randomly with respect to the degree of relatedness, indicating a lack of inbreeding avoidance by selective mating. Snails that mated with full-sibs did not differ in number of eggs, hatching success of eggs, or number of offspring produced from those mated with unrelated conspecifics. In another breeding experiment with *A. arbustorum* from a different population, Chen (1993) compared the reproductive performance of snails that mated with full-sibs to that of snails that mated with unrelated partners. Again, there was no difference in the number of eggs produced. However, eggs of inbred snails showed a lower hatching success (30.4%) than those of outbred snails (48.5%). Thus, inbred snails had fewer hatchlings. Furthermore, inbred offspring reared in the garden had a higher mortality rate than outbred offspring reared in the same environment, but no difference was found when offspring from both groups were kept in the laboratory. This result supports the hypothesis that cross-fertilization in simultaneous hermaphrodites is maintained by inbreeding depression. It also shows that the extent of negative inbreeding effects vary among populations and environments in which the snails are kept.

MULTIPLE PATERNITY IN THE WILD

The genetic background of shell polymorphism, including ground color and banding pattern, is well studied in *Arianta arbustorum* (Oldham 1934, Cook and King 1966). One locus controls the ground color of the shell, which may be yellow or brown (brown being dominant). Thus, shell color can be used as a genetic marker to analyze paternity in

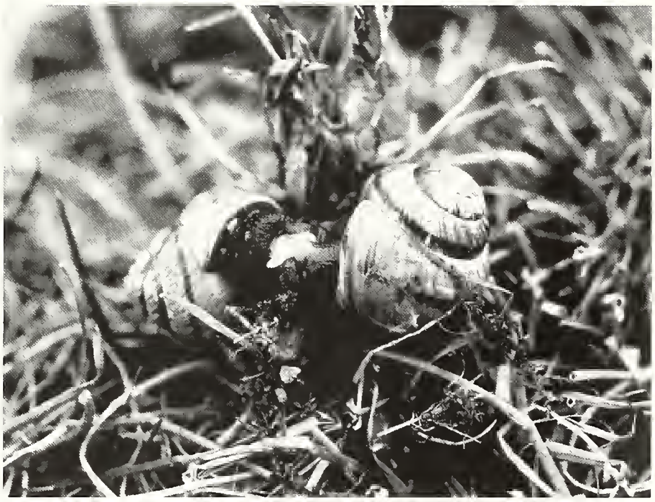

Figure 1. Copulating pair of *Arianta arbustorum* in an alpine grassland in Switzerland (photo: B. Baur).

broods of double-mated individuals of known genotype because shell color is already distinctly expressed in hatchlings. Twenty-two adult *A. arbustorum* with yellow shells were collected in a pasture on Mt. Raimeux (Jura mountains, Switzerland) at an elevation of 1290 m (B. Baur 1994b). This population is dimorphic with respect to shell color. Two estimates revealed very similar results: 27.7% (n = 94) of the adult *A. arbustorum* had yellow shells on 20 May 1993 and 27.4% (n = 124) on 24 July 1993. Assuming Hardy-Weinberg equilibrium, the frequency of the allele "brown" is estimated to $p = 0.475$ and that of the allele "yellow" to $q = 0.525$. Of the 22 adult *A. arbustorum*, 19 produced 34 clutches with fertilized eggs in the laboratory. Nine of the 34 clutches (26.5%) deviated significantly from Mendelian ratios of single paternity, providing evidence for multiple paternity (B. Baur 1994b). This figure may underestimate the actual frequency of multiple paternity because repeated matings with snails of the same genotype will produce results that are indistinguishable from the broods of single matings. Several snails deposited 2 or 3 clutches. Considering the total number of offspring produced by single snails during 65 days, the progeny of 12 of 19 individuals (63.2%) deviated significantly from Mendelian ratios of a single copulation.

SPERM COMPETITION

Sperm competition, the competition between spermatozoa from different males to fertilize the eggs of a single female during one reproductive cycle, is a form of male-male competition that occurs between insemination and fertilization (Parker 1970). It is a part of intrasexual selection. Sperm competition has been solely treated as a male-male conflict over many years, with females being an inert arena in which the conflict occurs. More recently, female processes that affect male reproductive success and that occur after copulation have received increasing attention: cryptic female choice and selective sperm use (Eberhard 1996, see below).

Courtship and dart shooting

During courtship, many helicid snails attempt to pierce the body walls of their mating partners with mucus-coated calcareous darts (Koene and Schulenburg 2005). The mucus covering the dart induces conformational changes in the female reproductive tract of the recipient, closing off the entrance to the gametolytic bursa copulatrix. In *Helix aspersa* O. F. Müller, 1774, a pulmonate land snail with obligate dart shooting, individuals that were hit by darts stored significantly more sperm than did snails that were missed (Landolfa *et al.* 2001, Rogers and Chase 2001, 2002, Landolfa 2002, see also Chase 2007).

In *Arianta arbustorum*, dart shooting is not an obligatory element of courtship behavior. Baminger *et al.* (2000) examined dart shooting in relation to different aspects of sperm competition (allosperm storage and autosperm delivery) in three natural populations of *A. arbustorum* in the Eastern Alps, Austria. Twenty-six (30.2%) of the 86 copulating snails used their darts. There was no reciprocity in dart shooting: individuals shot their darts independently of the behavior of their mating partners. Further, the occurrence of dart shooting was related neither to the number of sperm delivered nor to the number of sperm received from the partner. Finally, the occurrence of dart shooting was not influenced by the amount of allosperm stored from previous matings. In laboratory matings of *A. arbustorum* from Mt. Raimeux, Swiss Jura mountains, 50% of the copulating individuals pushed or tried to push a dart into their partners (Bojat and Haase 2002). Dart recipients did not store more sperm than snails not hit by the dart. This indicates that the importance of dart shooting for sperm storage varies among species of snails and even among populations of the same species.

Spermatophore formation and sperm transfer

In *Arianta arbustorum*, the spermatophore has a distinctive form consisting of head filament, sperm container, and a 2-3 cm long tail. The spermatophore is formed in the epiphallus (head filament and sperm container) and in the flagellum (tail) during copulation (Hofmann 1923). Baminger and Haase (2001) examined formation and filling of spermatophores by collecting spermatophores in mating pairs of *A. arbustorum* at certain intervals after the beginning of copulation. Two minutes after penis intromission there was no trace of a spermatophore. After 5 min, a spermatophore without tail was visible in two of four individuals. After 10 min, each snail contained a complete, albeit small, spermatophore consisting of head filament, container, and fully-developed tail. The part of the spermatophore that contained the sperm was increasing in size until shortly before the spermatophore was transferred (approx. 90 min after penis intromission). Spermatophore formation was initiated more or less synchronously in mating partners shortly after the beginning of copulation. However, the growth and final size of the spermatophore were not adjusted between the mating partners. These observations are supported by the finding that the numbers of sperm transferred by two mating snails are not correlated (Baur *et al.* 1998). This also indicates that sperm trading (sensu Leonard 1991) does not occur in *A. arbustorum* (see also number of sperm delivered).

The form of the spermatophore in *Arianta arbustorum* is very similar to that in *Helix pomatia* (Lind 1973), except that the head of the spermatophore is proportionally shorter and not filamentous in the latter species (Hofmann 1923). The tail, with its spiral cross section, especially suggests that the function is identical in both species (Baminger and Haase 2001). After spermatophore transfer, only sperm that migrate through the tail, which reaches deep into the vagina, can bypass the digesting bursa copulatrix and reach the spermoviduct through which they travel to the spermatheca, where they are stored. The head filament probably guarantees that the spermatophore is not pushed too far up into the diverticulum, preventing the tail from getting too close to the entrance of the bursal duct. The dissolution of the spermatophore in the female tract of the receiver takes more than one week in *A. arbustorum* (Haase and Baur 1995).

Number of sperm delivered

Sperm number, in some cases, is an important determinant for achieving successful fertilization in sperm competition (Birkhead and Møller 1998). Theoretical models and empirical evidence from various studies suggest that, fundamentally, numerical superiority is an adaptive strategy for sperm competition (Parker 1990a, 1990b, Birkhead and Møller 1998). However, males may incur a substantial cost in the production of ejaculates and spermatophores (Dewsbury 1982, Nakatsuru and Kramer 1982). Parker's model (1990a, 1990b) predicts that males faced with an increased risk of sperm competition should maximize the prospects of fertilization by inseminating more sperm per ejaculate. For example, owing to sperm storage from previous copulations, mating with a nonvirgin partner may result in a higher risk of sperm competition than mating with a virgin partner. Experimental evidence for adjustment of ejaculate size with respect to mating history has been provided for insects, salamanders, rats, and humans (Birkhead and Møller 1998). In other species, however, males do not adjust the size of their ejaculates to the mating history (*e.g.*, in zebra finches, Birkhead and Møller 1998).

An adjustment of the number of sperm released is also a prerequisite for sperm trading. In simultaneous hermaphrodites, a sexual conflict may arise when there is a difference in potential fitness gain between the role of the sperm donor and that of the sperm receiver (Charnov 1979, Leonard 1991). Hermaphroditic individuals in a population would benefit from mating primarily in the more fitness-enhancing sexual role, leading to a conflict of interest between two prospective mating partners (Charnov 1979). Gamete trading might have evolved to resolve the sexual conflict in simultaneous hermaphrodites (Leonard 1991). The gamete-trading model is based on the premise that the preferred role for a simultaneous hermaphrodite will be the one that controls fertilization (Leonard 1991). In particular, this model predicts that where the female function controls fertilization, the mating system will be based on sperm trading.

Baur *et al.* (1998) examined whether individuals of *Arianta arbustorum* adjust sperm release according to the potential risk of sperm competition incurred with a virgin or nonvirgin mating partner and whether sperm trading occurs in mating pairs. In controlled mating trials, focal snails were allowed to copulate with virgin or nonvirgin partners to simulate a different risk of sperm competition in a given mating. The number of sperm transferred ranged from 802,620 to 3,968,800 (mean = 2,185,100; $n = 91$), but was related neither to the mating history of the partner nor to the duration of the copulation. This indicates that individuals of *A. arbustorum* are not able to adjust sperm expenditure to the mating history of the partner. Furthermore, the number of sperm transferred was correlated neither with the size of the donor nor with the size of the recipient. There was, however, a high degree of reciprocity in spermatophore transfer: in 45 of the 46 mating pairs investigated both partners delivered a spermatophore that contained spermatozoa. In contrast, the numbers of sperm transferred by the two mating partners were not correlated. This indicates that sperm trading does not occur in *A. arbustorum*.

Locher and Baur (2000a) examined the effect of increased risk of sperm competition on male and female reproductive traits in individuals of *Arianta arbustorum*. In a laboratory experiment snails were exposed to mucous trails of conspecifics, simulating a high risk of sperm competition. Courtship behavior, spermatophore size, and number of sperm delivered were not influenced by a higher risk of sperm competition. However, snails constantly exposed to mucous trails of conspecifics deposited more egg batches than snails denied any cues from conspecific mucous trails. This indicates that *A. arbustorum* does not respond to experimentally increased cues from conspecifics, which were assumed to mimic a high risk of sperm competition by delivering more sperm.

Number of matings and intermating interval

Multiple mating is common in helicid snails. Individuals of *Helix pomatia*, *Cepaea nemoralis*, and *Arianta arbustorum* have been observed to mate repeatedly with different partners in the course of a reproductive season, resulting in multiple-sired broods (Wolda 1963, Murray 1964, B. Baur 1988b, 1994b). Individuals of *Helix pomatia* copulated 2-6 times per year in a Danish population (Lind 1988) and 2-4 times in a German population (Tischler 1973). Individuals of *H. aspersa* copulated on average 3 times (maximum 7 times) in a British population (Fearnley 1993, 1996). Actual records on the number of matings in natural *A. arbustorum* populations are not available. Video-recording of *A. arbustorum* kept in groups of six snails under laboratory conditions revealed that individuals copulated between 0 and 3 times in a period of 58 days (N. Minoretti, pers. comm.). Furthermore, paternity analysis in egg batches of *A. arbustorum* sampled in a natural population indicated that at least 63% of the snails used sperm from two or more mates to fertilize their eggs (B. Baur 1994b).

The few data available on mating frequency in gastropods suggest that terrestrial gastropods copulate less frequently than freshwater and marine gastropods (Baur 1998). In intertidal and terrestrial gastropods the reproductive activity is limited by favorable environmental conditions. The long-lasting courtship and mating behavior of terrestrial gastropods may exceed the period favorable for locomotory activity (the high risk of desiccation may incur a significant cost of mating). Freshwater and marine habitats, however, may provide temporally more constant conditions favorable for mating activities than terrestrial habitats. Other explanations for the relatively small number of copulations in terrestrial pulmonates include the costs of producing mucus during mating, the production of spermatophores and darts (in some species), and the large number of sperm delivered during a copulation, which may result in sperm depletion.

Locher and Baur (1999) showed that individuals of *Arianta arbustorum* needed at least 8 days to replenish their sperm reserves after a successful copulation. Furthermore, the number of sperm delivered in the second copulation increased with an increasing intermating interval from 6 to 29 days. This finding suggests that the number of sperm delivered increases with even longer intermating intervals. Hänggi *et al.* (2002) examined the size of the spermatophore and the number of sperm delivered in two groups of *A. arbustorum* that remated either after 3-4 weeks or after 7-8 weeks. The results indicated that *A. arbustorum* entirely replenishes its autosperm reserves within 3-4 weeks after a successful copulation.

Sperm size and quality

Much interest has also been focused on theory concerning the significance of the size and quality of sperm (*e.g.*, Parker 1982, Parker and Begon 1993, Pizzari and Birkhead 2002). The size of sperm may influence their power and swimming speed as well as longevity because of changes in the energetic demands of longer or shorter flagella. For example, in echinoids that use broadcast spawning, there is evidence that sperm velocity and longevity covary between and within species (Levitan 1993). Levitan (2000) has shown that in a sea urchin, sperm velocity and longevity are traded off against each other. In taxa with sperm storage organs, sperm length may determine the ability to reach the storage organs first and to move to the ovum from the storage organs once ovulation takes place. However, assuming a fixed resource budget, smaller sperm may allow males to produce more gametes, which may be adaptive if sperm compete numerically (Parker 1982). Confounding variables, such as the morphology and biochemistry of the female reproductive tract, might also affect sperm form and function.

Besides the size and number of sperm transferred, the quality of ejaculates (proportion of live, morphologically normal spermatozoa and motility of spermatozoa) might be important in determining the outcome of sperm competition. Indeed, recent studies in gonochoristic animals reveal substantial intraspecific variation in sperm motility and longevity. This variation may function in postcopulatory sexual selection. A synthesis of the available literature indicates that these sperm-quality traits affect fertilization success and that they are important in both sperm competition and cryptic female choice (Snook 2005).

Molluscan sperm are characterized by a remarkable array of morphological features (Thompson 1973, Anderson and Personne 1976, Hodgson *et al.* 1996). The interspecific variation in sperm morphology is frequently used as a taxonomic character. Data on sperm length are available for several gastropod species. Spermatozoa of terrestrial pulmonates are among the longest of the molluscs (*e.g.*, 850 µm in *Helix pomatia*; Thompson 1973). However, intraspecific variation in sperm length and quality has not been analyzed in any hermaphroditic gastropod species.

Minoretti and Baur (2006) developed techniques to measure sperm length, sperm velocity, percentage motility, and longevity of sperm in *Arianta arbustorum*. They examined variation in sperm length in individuals from four natural populations and variation in velocity, motility, and longevity of sperm in two populations. Sperm of *A. arbustorum* are monomorphic. Like other pulmonates, *A. arbustorum* produces extremely long sperm. Independent of shell size, sperm length differed among populations (mean values of populations: 878, 898, 913, and 939 μm) and—to a minor extent—even among individuals within populations. Mean sperm length of a snail was not correlated with the number of sperm delivered in a spermatophore. Individual snails showed consistent sperm length in successive matings. The mean sperm velocity was not influenced by shell size, nor did it differ between populations. However, mean sperm velocity differed among individual snails (range 52-112 μm/s). Percentage motility and longevity of sperm differed between snails from different populations but were not affected by shell size. No correlations were found between length, velocity, percentage motility, and longevity of sperm. Thus, individual snails differed in sperm quality. This interindividual variation may partly explain differences in fertilization success.

Sperm precedence

Sperm precedence is the differential sperm usage from consecutive matings (mating order effect). It is typically measured as the proportion of eggs fertilized by the second of two mates (the P_2 value). Patterns of sperm utilization were investigated in double-mated individuals of *Arianta arbustorum* (B. Baur 1994b). In particular, the effects of delay between copulations (range 9-380 days) and size of the sperm donor on sperm precedence (P_2) were examined. Using shell color as a genetic marker, paternity was analyzed in 132 broods produced by 35 snails that had mated with two partners of different genotypes. Sperm precedence (P_2) was influenced by the time between the two matings when the mating delay exceeded 70 days (one reproductive season). In the first brood of snails that mated twice within 70 days, P_2 averaged 0.34, indicating precedence of sperm from the first mate. In contrast, P_2 averaged 0.76 in broods of snails that remated in the following season, indicating a decreased viability of sperm from the first mate. The size of sperm-donating individuals had no effect on the fertilization success of their sperm in the first brood produced after the second copulation. Analysis of long-term sperm utilization in 23 snails that laid 3-9 batches over 2 years revealed striking differences among individuals. Five snails (21.7%) exhibited precedence of sperm from the first mate throughout, 8 snails (34.8%) showed precedence of sperm from the second mate throughout, whereas 10 snails (43.5%) exhibited sperm mixing in successive batches. This indicates that different mechanisms might be involved in creating the observed inter-individual variation in sperm precedence.

FEMALE ROLE IN SPERM COMPETITION: CRYPTIC FEMALE CHOICE

Until recently, most research concentrated on male aspects of sperm competition in gonochoristic animals. In the past few years, there has been increasing interest in the possibility that females influence the outcome of sperm competition by cryptic female choice and selective sperm use (Eberhard 1996). Females might be able to discriminate between and differentially utilize the sperm of different males, a process referred to as 'sperm choice' (Birkhead 1998). There are broad and narrow definitions of "sperm choice"; some authors make it synonymous with "cryptic female choice" (see Eberhard 2000, Kempenaers *et al.* 2000, Pitnick and Brown 2000). Cryptic female choice has been defined as nonrandom paternity biases resulting from female morphology, physiology or behavior that occur after coupling (Pitnick and Brown 2000). This definition ascribes to sperm choice any biases in paternity owing to the way females handle sperm, regardless of the specific mechanism or evolutionary causes, and regardless of proximate control. The only relevant consideration for this definition is whether a female-mediated process generates sexual selection on males. A general problem with cryptic female choice is that it is difficult to rule out the direct influence from males (*e.g.*, Edvardsson and Arnqvist 2000).

In simultaneously hermaphroditic pulmonates the role of the female duct is to receive sperm from a copulating partner, to store the sperm, to provide a site for fertilization, to form the egg capsule, and to digest sperm and remnants of the spermatophore so as to absorb nutritional fluids received with the ejaculate.

Variation in the morphology of the female reproductive tract

Rapid evolution of reproductive traits has been attributed to sexual selection arising from interactions between the sexes (*e.g.*, Eberhard 1996). Inter- and intraspecific studies in gonochoristic animals revealed a covariation between sperm characteristics and the size of the female reproductive tract, indicating an evolutionary divergence, which is consistent with the theory of post-copulatory sexual selection. In *Arianta arbustorum*, like other terrestrial pulmonates, the enormous variation in structure and morphology of the spermatheca, fertilization chamber, and sperm digesting organ could have evolved in response to different levels of sperm competition and/or cryptic female choice (Baur 1998). Baminger and Haase (2000) examined the variability of the distal genitalia involved in spermatophore production, reception, and manipulation in 113 adult individuals of *A. arbustorum* from six natural populations. In particular, Baminger and Haase (2000) asked whether the variation in genitalia is related to the intensity of sexual selection, measured as local population density. The size of the genitalia was unexpectedly inversely related to population density, probably because of an increased inhibitory effect of snail mucus. Patterns of variation of female and male characters did not differ. However, the influence of sexual selection on genitalia size and variance could not be unambiguously determined.

Beese *et al.* (2006a) quantified the variation in male and female reproductive traits among six natural populations of *Arianta arbustorum* and examined the covariation in interacting traits. There was a significant among-population variation in spermatophore volume, number of sperm transferred, and sperm length, as well as in volume of the sperm storage organ (spermatheca) and number of tubules, but not in spermathecal length. Furthermore, there was no relationship between sperm size and spermathecal length. There was, however, a positive association between the number of sperm transferred and spermathecal volume. This result suggests that the same post-copulatory mechanisms that operate in gonochorists drive the correlated evolution of reproductive characters in hermaphrodites.

The wall of the spermatophore received is dissolved in the bursa tract diverticulum (Beese *et al.* 2006b). The digested material is taken up by epithelial cells.

Organ for sperm storage

Individuals of *Arianta arbustorum* are able to store viable sperm from different mating partners for more than 1 year (Baur 1988b). The morphology of the sperm storage organ (spermatheca) may influence the outcome of sperm competition in *A. arbustorum*, as shown in insects (Simmons and Siva-Jothy 1998). *Arianta arbustorum* shows a considerable variation in the structure of the spermatheca (Haase and Baur 1995, Beese and Baur 2006). It consists of two to nine blind tubules uniting to a common duct, which opens into the fertilization chamber (Haase and Baur 1995, Baminger and Haase 1999, Baminger *et al.* 2000). The musculature surrounding the spermathecal tubules is arranged in a complex three dimensional network (Bojat *et al.* 2001a, 2001b, 2001c). If there were a selective activation of the muscles of each tubule (which has not yet been examined), this would allow the animal to expel sperm stored in single tubules and thus promotes a selective fertilization of eggs. The ciliation of the common duct is probably responsible for the distribution of incoming sperm among the tubules.

Bojat and Haase (2002) assessed the amount of allosperm stored in the spermatheca in relation to the structure of the spermatheca (number of spermathecal tubules) in 18 individuals of *Arianta arbustorum* that had copulated once. Snails differed in patterns of sperm storage: two individuals used 100% of their spermathecal tubules, two used 80%, three 75%, two 66.7%, one 50%, two 40%, three 33.3%, two 25%, and one used 20%. The main tubule always contained sperm (51-100% of the total amount of sperm stored, *i.e.*, more than all lateral tubules combined). The amount of sperm stored was not correlated with the volume of the received spermatophore. However, the amount of sperm stored was positively correlated with the number of spermathecal tubules. This suggests that the female role of the receiver controls the number of sperm stored.

Baminger and Haase (1999) examined whether the variation in number of spermathecal tubules and the amount of allosperm stored are influenced by the risk of sperm competition, as indicated by the local density of adult *Arianta arbustorum* in six natural populations in the Eastern Alps, Austria. The number of spermathecal tubules ranged from two to nine. However, the six populations did not differ in either the mean number of spermathecal tubules or the cumulative length of the tubules. Individuals from different populations did not differ in the amount of sperm stored, and the amount of sperm stored was not correlated with population density. This indicates that the risk of sperm competition does not affect the number of spermathecal tubules. However, it is still not known whether individuals in high-density populations store allosperm from more different mating partners than those in low-density populations.

A histochemical analysis of the spermatheca of *Arianta arbustorum* revealed polysaccharides in the periphery of connective tissue and muscle cells (Bojat *et al.* 2003). Polysaccharides were differentially distributed in the epithelial cells of the fertilization chamber and spermatheca, indicating regions that differed in physiological activity. In general, the concentration of polysaccharides including glycogen increased towards the blind end of the spermathecal tubules. Lipids were more or less equally distributed in the epithelium along the tubules. Polysaccharides and lipids have nutritive function. The highly active epithelium could provide nutrients for the stored and active spermatozoa (Rogers and Chase 2002). However, an exchange of substances between epithelium and spermatozoa has not been observed.

BENEFITS OF MULTIPLE MATING FOR FEMALES

In many animals, males are selected to mate as many times as possible to maximize their reproductive success (Bateman 1948, Trivers 1972). For females, in contrast, the advantage of multiple mating is not so obvious. The relatively small number of ova produced by a female could be fertilized by sperm from one or very few male ejaculates, especially when the female can store sperm or has a short reproductive period. Moreover, possible costs of mating should select against unnecessary matings. While females of some species mate only once in their lives, multiple mating by females is generally very common. Several hypotheses have been suggested to explain the adaptive significance of multiple mating by females (Birkhead and Møller 1998). Among them, the hypothesis of sperm replenishment is the most straightforward explanation for multiple mating by females. The underlying mechanism could vary: the sperm received from one male may not be enough to fertilize all the eggs produced by a female, the viability of sperm stored may decrease with time, or a mate may have transferred sperm of low quality. Another hypothesis predicts genetic advantages (multiple mating with different partners may lead to multiple paternity and thus increase the genetic variability among the offspring of a brood). Furthermore, females may enjoy nutritional benefits from repeated matings by receiving nutrients with the spermatophore. These hypotheses are not mutually exclusive.

Chen and Baur (1993) examined reproductive traits over two years in individuals of *Arianta arbustorum* that copulated several times per year (snails kept in pairs), in individuals that copulated twice (once at the beginning of each year) or once (at the beginning of the first year), and in individuals prevented from copulation (snails kept isolated). Copulations were not always reciprocally successful: 3 of 57 snails (5.3%) failed to produce fertile eggs although their mates reproduced successfully. Similarly, 2 of 15 pairs (13.3%) failed to reproduce successfully. Snails allowed to mate repeatedly within each season tended to lay more eggs than snails that mated once per year. However, the number of hatchlings did not differ significantly between the two treatment groups because eggs laid by snails allowed to mate repeatedly had a lower hatching success. Snails that remated in the second year laid more eggs with a higher hatching success, and thus produced more hatchlings, than snails that mated only once at the beginning of the first year. Snails that were prevented from mating produced a few hatchlings (by self-fertilization) in the second year; their reproductive success was less than 1% of that of mated snails. These results suggest that multiple mating is also adaptive for the female function of *A. arbustorum* by increasing female fecundity and fertility and serving as a hedge against unsuccessful copulations.

Egg production in several species of stylommatophoran gastropods is stimulated by mating behavior and/or substances derived from male ejaculate (Takeda 1983, Bride *et al.* 1991). In *Helix aspersa*, mating increases the synthesis and release of a dorsal body hormone essential for vitellogenesis, ovulation, and egg-laying (Saleuddin *et al.* 1991). Whether this activation of the dorsal body is direct, under either neural or hormonal control, or indirect under gonadal influence, is not known (Saleuddin *et al.* 1991). B. Baur and A. Baur (1992b) examined experimentally whether extended courtship display or repeated copulation in the course of a reproductive season stimulates egg production in *Arianta arbustorum*. Clutch size decreased in successive egg batches of individuals that copulated once at the beginning of the reproductive season (a seasonal decrease in clutch size was also observed in *A. arbustorum* kept in field cages; Baur 1990a). Repeated copulation, however, was found to increase clutch size, while courtship display did not affect egg production. Repeated copulation neither accelerated the onset of egg laying nor increased the hatching success of eggs. These results suggest that reciprocal intromission and/or receipt of a spermatophore, but not the long-lasting courtship behavior, stimulates egg production in *A. arbustorum* (B. Baur and A. Baur 1992b).

MATERNAL INVESTMENT AND EGG PROVISIONING

Parental investment may often be critical to the survival and growth of young, but the larger the investment per offspring, the lower the number of offspring that can be produced. Several models have been developed to predict the optimal size of offspring under different environmental conditions. Although the models make different predictions, these are based on the assumption that egg size is a reliable measure of the amount and quality of resources invested in each offspring (*i.e.*, larger eggs are supposed to contain more organic material). A. Baur (1994) examined the within- and between-clutch variation in egg size and nutrient content of *Arianta arbustorum*. The volume of single eggs ranged from 5.5 to 26.3 mm^3 (grand mean 13.4 mm^3). The overall range of the nitrogen concentration of the eggs was 3.1-5.0% (grand mean 4.1%), and that of the carbon concentration 28.6-34.9% (32.2%). The nitrogen concentration indicates that eggs of *A. arbustorum* have a protein concentration of 25.5%. The within-clutch variation in egg size expressed by the coefficient of variation averaged 11.1% for egg volume and 8.3% for dry weight. Corresponding values for the concentrations of N and C were 3.7 and 1.6%. Thus, egg size was in general more variable than the nutrient concentration of the eggs. Considering mean clutch values, the nutrient contents (in mg) scaled isometrically with egg size.

Successive studies showed that not only do egg and clutch size vary seasonally but also the protein and carbon concentrations of the eggs do so (A. Baur and B. Baur 1997). Furthermore, seasonal changes in egg size and egg provisioning occur among populations (Baur and Baur 1998).

Apart from increasing egg size, maternal nutrition can be enhanced by providing hatchlings with food. Alexander (1974) suggested that if parents were unable to increase their investment in young through increasing egg size, an alternative strategy is to increase clutch size and allow some siblings to consume others (the icebox effect). The optimum clutch size can be found by calculating the clutch size leading to maximum brood productivity, taking into account the effects of sibling cannibalism and possible trade-offs.

Maternal provision of trophic eggs of different types to hatchlings is widespread among marine gastropods. Numerous species of prosobranch snails normally produce trophic eggs, which serve as the first food for their progeny (B. Baur 1992b, 1994c). The consumption of trophic eggs can be facultative (may not occur in all egg capsules of a species) or obligate. A similar form of food provisioning has evolved in terrestrial gastropods. Hatchlings of various species of herbivorous land snails cannibalize sibling eggs (Baur 1992b). Emerging juveniles of *Arianta arbustorum* first eat their own egg shells and then the eggs of unhatched siblings, including those with fully developed embryos (Baur and Baur 1986). Egg cannibalism occurs exclusively during the hatchling stage (due to an age-specific occurrence of digestive enzymes), juvenile and adult snails being herbivorous (Baur 1987a). Cannibalistic hatchlings eat only conspecific eggs (Baur 1988c, 1988d) and do not discriminate between sib and non-sib eggs (*i.e.*, eggs from neighboring batches; Baur 1987b). Furthermore, newly hatched snails discriminate neither between fertilized and unfertilized conspecific eggs nor between eggs with well-developed embryos and eggs with less advanced embryos (Baur 1993b). Significant benefits accrue to cannibalistic hatchlings of *A. arbustorum*. Laboratory experiments demonstrated that newly hatched snails fed a cannibalistic diet during their first 10 days of life increased in wet weight 2.6 times as much as siblings fed on lettuce (Baur 1990b). In this experiment, egg consumption within 10 days ranged from 0.7 to 4.0 eggs per individual and increase in weight of cannibalistic hatchlings was positively correlated with the number of eggs consumed. Diet did not affect hatchling survival during the first 10 days, but it did influence future survival: 66.6% of the individuals initially fed on eggs attained adulthood compared to 38.0% of those fed on lettuce. Cannibalistic hatchlings tended to complete shell growth more rapidly and thus became sexually mature earlier than non-cannibalistic ones, but the two groups did not differ in adult shell size. Thus, a cannibalistic diet during the hatchling stage will give accelerated growth and higher survival.

The extent of within-batch egg cannibalism depends primarily on the hatching asynchrony of the eggs and on the hatchlings' propensity for cannibalism (Baur and Baur 1986, B. Baur 1994c). Under natural conditions, the hatching asynchrony and, as a consequence, the extent of egg cannibalism will depend also upon the type of oviposition (batches or scattered eggs), on the spatial heterogeneity of egg-laying sites, and on climatic conditions. The great variation between populations in propensity for cannibalism suggests different costs and benefits of egg cannibalism in different situations (B. Baur 1994c).

SEX ALLOCATION

Reproductive resource allocation is a fundamental aspect of life history with profound ecological and evolutionary consequences. Allocation decisions in hermaphroditic plants and animals are particularly interesting because individuals can potentially maximize reproductive success through a wide variety of different strategies. Thus, a key observation for testing sex allocation theory in simultaneous hermaphrodites is the proportion of resources devoted to male vs. female function (Charnov 1982). The specific allocation strategy followed by a hermaphrodite may affect the extent of sexual selection and mating behavior (Michiels 1998). Most models of sex allocation are based on the concept of male and female gain curves (the relationship between relative investment in either male or female gamete production and resulting reproductive success; Charnov *et al.* 1976, Charnov 1982). These models have received substantial empirical support (*e.g.*, in coral reef fishes [Fisher 1981, Fischer and Petersen 1987] and in a polychaete worm [Sella 1990]. All these hermaphrodites have external fertilization. Many hermaphroditic invertebrates, however, have some form of copulation, sperm storage, and internal fertilization (Michiels 1998).

More recently, models of sex allocation for outbreeding hermaphrodites with internal fertilization and sperm storage have been developed (Charnov 1996, Greeff and Michiels 1999b). These models consider how various aspects of sperm competition, such as mating frequency, sperm digestion, and different mechanisms of sperm displacement affect sex allocation in simultaneous hermaphrodites. The models predict that a reduced mating rate leads to a reduction in resources allocated to the male function (Charnov 1996, Greeff and Michiels 1999b), while sperm digestion leads to an increase in allocation to the male function (Greeff and Michiels 1999b).

Locher and Baur (2000b) examined the effect of mating frequency on male and female reproductive output (number of sperm delivered and eggs deposited) and on the resources allocated to the male and female function (dry mass, nitrogen, and carbon contents of spermatophores and eggs) in individuals of *Arianta arbustorum*. Virgin snails were allowed to mate once, twice, or three times within a period of 51 days. Snails from the three treatment groups did not differ in shell volume. The total number of sperm delivered increased from 3,098,000 in snails that copulated once to 5,001,000 in snails that copulated twice, and to 6,849,000 in individuals with three copulations. Considering the number of sperm delivered per copulation, however, there was no difference between snails with different numbers of copulations. Female reproductive output was not influenced by the number of copulations. Snails that copulated once, twice, or three times produced the same number of egg batches (3.9, 3.8, and 4.3, respectively) and the same number of eggs (113.7, 116.2, and 116.3) within one reproductive season. Considering snails from all treatment groups, there was a positive correlation between the total number of sperm delivered and the number of eggs produced. This indicates that individuals that delivered many sperm generally produced a large number of eggs.

In this experiment, the dry mass of spermatophores transferred averaged 0.91 mg (Locher and Baur 2000b). Spermatophores had a nitrogen concentration of 12.05%, indicating that a spermatophore on average contained 0.11 mg nitrogen. The dry mass of single eggs averaged 2.14 mg with a nitrogen concentration of 4.02%. Thus, one egg on average contained 0.086 mg nitrogen. The relative allocation to the male function (expressed as percentage of the total dry mass or amount of nitrogen devoted to the male function) increased with increasing number of copulations (dry mass: 0.58% in snails that copulated once, 0.91% in snails that copulated twice, and 1.66% in individuals that copulated three times; nitrogen: 1.72%, 2.68%, and 4.72%, respectively). However, independently of the measure chosen, reproductive allocation was highly female-biased. In none of the measures used did the average proportion of resources devoted to the male function reach 5% of the total allocation in snails that copulated three times. Considering individual snails, the maximum nitrogen allocation to the male function was 13.35% in snails that copulated three times. Thus, only a minor part of the resources available for reproduction were devoted to the male function. Furthermore, snail size did not affect the relative reproductive allocation to male or female function. The finding that an increased mating frequency leads to an increased allocation to the male function was predicted by Charnov (1996) and Greeff and Michiels (1999b), even though their models considered much higher mating frequencies (5-50 or even an infinite number of copulations). Taking into account the short period of activity of snails living in subalpine populations, the assumed range of 1-3 copulations per reproductive season might be reasonable for the animals studied.

The female skew in sex allocation found in *Arianta arbustorum* was much larger than predicted by the models of Charnov (1996) and Greeff and Michiels (1999b). A possible explanation for this pronounced female skew could be incomplete estimates of reproductive allocation to either function. In simultaneously hermaphroditic land snails like *A. arbustorum*, each mating in the male role carries costs, which include the cost of courtship behavior (*e.g.*, the optional dart shooting), spermatophore and sperm production, and other possible costs associated with mating. During the long-lasting courtship, gastropods produce huge amounts of mucus, an energetically expensive behavior (Davies *et al.* 1990). To my knowledge no numerical estimate of the costs of courtship behavior is available for any terrestrial gastropod. Furthermore, it is not clear whether the costs of courtship can be entirely assigned to the male function. For several reasons repeated mating might also be advantageous for the female function (see above).

A fundamental assumption of sex allocation theory in simultaneous hermaphrodites is a trade-off between male and female function, *i.e.*, the animal has a fixed amount of resources to allocate between the genders (Charnov 1982). Locher and Baur (2000a, 2000b) did not find any trade-off between the two functions. In contrast, a positive relationship between the resources allocated to the male and female functions was recorded. This result could be explained by a condition-dependent allocation and/or a "good genes" scenario. With regard to quality, any genes that affect the quality of spermatozoa and ova in the same direction would lead to a positive association between the two (Schlichting and Delesalle 1997). In hermaphroditic pulmonates such as *Arianta arbustorum*, spermatozoa and ova are produced simultaneously in the same organ, the so-called ovotestis. It would be most interesting to disentangle possible associations between the quality of spermatozoa and ova in these snails.

Trade-offs in resource allocation may not occur or may be less pronounced under favorable conditions. Under stressful conditions, such as limited food supply, high temperature, or drought, the energy intake might be carefully shared among different functions, including reproduction. Locher and Baur (2002) examined the effect of nutritional stress on mating behavior and male and female reproductive output (dry mass and nitrogen contents of spermatophores, sperm delivered, and eggs deposited) in individuals of *Arianta arbustorum* kept under three different food regimes: ample (100%), restricted (50%), and extremely restricted (25%) food supply. Independent of the extent of nutritional stress, 10-12% of the resources taken up were invested in reproductive output (both gender functions together) and 88-90% in maintenance (including feces and excretion). Courtship and copulation behavior was affected by nutritional stress. Except for one pair, snails with an extremely restricted food supply did not mate. Individuals with restricted food supply tended to court longer and copulated for a shorter period than individuals with ample food supply. Nutritional stress did not affect the number of sperm delivered. However, snails with a restricted food supply produced fewer eggs. Thus, snails kept under nutritional stress invested relatively more resources in the male function than in the female function. Nevertheless, the absolute reproductive output remained highly female biased (>95% in all experimental groups).

In hermaphroditic snails a reduced supply of protein and calcium might affect growth and alter the allocation to either sexual function. Wacker and Baur (2004) tested this hypothesis by maintaining subadult *Arianta arbustorum* on artificial diets composed of single compounds (particular amino acids, carbohydrates, fatty acids, minerals, vitamins) on an agar-based diet. Snails fed a high protein diet grew faster and reached adulthood earlier than individuals fed a low protein diet. Different calcium contents did not affect shell growth, but increased mortality when the calcium content of the food was low. Furthermore, diet-related differences in mating propensity were found.

CONCLUDING REMARKS

Much recent research effort has been directed at explaining sex-specific differences in reproductive strategies and sexual selection in gonochoristic animals. Simultaneous hermaphroditism imposes evolutionary constraints on reproducing individuals that are different from those in gonochoristic species. Yet, simultaneous hermaphrodites, in particular pulmonate gastropods, have received little attention with respect to mating strategies and sexual selection. This review summarizes the present knowledge of several aspects of sexual selection in *Arianta arbustorum*. Several other gastropod species may be well-suited for further studies on mating strategies and sexual selection. There are many topics that remain largely unexplored and there is much to be learned in this most interesting animal group.

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A literature database on the mating behavior of stylommatophoran land snails and slugs*

Angus Davison[1] and Peter Mordan[2]

[1] Institute of Genetics, School of Biology, University of Nottingham, Queens Medical Centre, Nottingham, NG7 2UH, UK, angus.davison@nottingham.ac.uk

[2] Department of Zoology, The Natural History Museum, London, SW7 5BD, UK, pbm@nhm.ac.uk

Abstract: Stylommatophoran land snails and slugs generally mate by shell-mounting or face-to-face. Although phylogenetic evidence suggests that the mating position has remained more or less constant throughout the evolution of most lineages, other aspects of mating behavior and associated reproductive characters are highly variable. Along with other gastropods, therefore, stylommatophoran land snails and slugs could be particularly useful in trying to understand sex and sex allocation theory in hermaphrodites. It is often difficult, however, to compare mating behavior in different species because the literature is difficult to access or reports have not been formally published. Here we review studies on the mating behavior of snails and slugs, with the additional aim of creating a central access point and database for use as a resource by those interested in stylommatophoran mating behavior. As we maintain the database, updated versions will be made available at http://www.molluscs.org.

Key words: love darts, mollusc, sexual conflict, reciprocal mating, simultaneous hermaphrodite

The mating behavior of a wide variety of stylommatophoran land snails has been observed, but the descriptions are often within texts that are not easily accessible, or cannot be searched electronically. Many malacologists have also made their own informal observations of mating behavior, but do not publish them for lack of time, or because they are not perceived to be of sufficient worth on their own. Because there have been no recent reviews of the mating behavior of snails and slugs, we set out to collect as many observations together as possible, both formal and informal. Such an approach has already proved useful in trying to understand how so-called "love" darts evolved (Davison *et al.* 2005). Differences in mating behavior have also been used to understand the distribution of chiral variation (or asymmetry) among different taxonomic groups (Asami *et al.* 1998) and the evolution of external sperm exchange (Emberton 1994). The aim of this brief review, therefore, is to create a starting point for a compilation of data on the mating behavior of stylommatophoran snails and slugs.

Although there are some exceptions (*e.g.*, the elongated penes and external fertilization of *Limax maximus* Linnaeus, 1758), mating in the majority of land snails and slugs can be classified as either face-to-face or shell-mounting (Figure 1; Asami *et al.* 1998). The vast majority of species are also simultaneous hermaphrodites. In theory, therefore, four different modes of mating are possible because sex is also either simultaneous reciprocal (both individuals are male and female at the same time) or unilateral (each individual has a defined role as male or female during a single round of mating):

Face-to-face, simultaneous reciprocal;
Face-to-face, unilateral;
Shell-mounting, simultaneous reciprocal;
Shell-mounting, unilateral.

When two individuals mate unilaterally, they often switch roles after one round of mating—male becomes female and female becomes male. The frequency with which this occurs is difficult to assess because it requires extended observations, and also the frequency of mate switching depends upon the condition (or desire) of each snail (Koene and Ter Maat 2005). The problem is further complicated because often the most efficient means to make laboratory observations of mating behavior is to isolate individuals for some time before bringing them together. For a variety of possible reasons (*e.g.*, availability of seminal fluid), isolated individuals are more likely to switch mates after one round of mating (Koene and Ter Maat 2005).

Another concern is whether sperm or spermatophore transfer is always reciprocal if mating is reciprocal; from an evolutionary point of view, the reciprocal exchange of sperm is just as important. In some species of *Succinea* Draparnaud,

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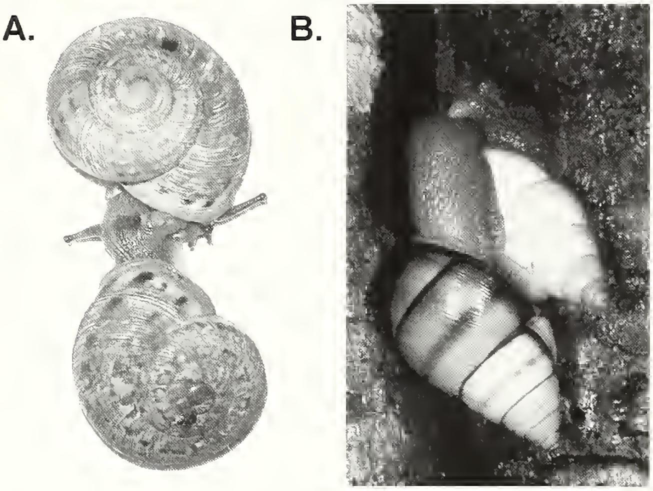

Figure 1. A, *Iberus marmoratus* Férussac, 1801 (Helicidae) mating face-to-face and simultaneous reciprocally. Horizontal field width approximately 6 cm. Photo by A. Davison, in the laboratory. B, *Liguus fasciatus* Pilsbry, 1912 (lower snail, acting as male) and *Orthalicus floridensis* Pilsbry, 1899 (upper snail, acting as female) (Orthalicidae) mating by shell-mounting. Specimens in hardwood hammock in the Redlands of southern Dade County, Florida. Image reproduced with the kind permission of the photographer, Phil Poland, ppoland1@tampabay.rr.com. Vertical field width approx. 7 cm.

1801 that mate reciprocally by shell-mounting, sperm transfer is sometimes unilateral (Jordaens *et al.* 2005), whereas other species such as *Arianta arbustorum* Linnaeus, 1758 have a high reciprocity (Baur 1998). In species that develop as males before becoming simultaneous hermaphrodites, such as *Lissachatina* (*Achatina*) *fulica* Bowdich, 1822, sperm transfer may frequently be unilateral (Tomiyama 1993).

We surveyed the formal literature on the mating behavior of land snails and slugs and then classified each species as to whether it mates face-to-face, by shell-mounting, simultaneous reciprocally, or unilaterally (Table 1). We also included informal observations, when available (mating position and, to a lesser extent, reciprocity can be scored from photographs), and tried to identify video recordings of mating behavior (Table 2). One striking, immediately apparent result is that face-to-face mating is exclusively associated with simultaneous reciprocal mating. Snails and slugs in three monophyletic groups mate face-to-face and simultaneous reciprocally: the Helicoidea, Limacoidea, and Philomycidae (Davison *et al.* 2005).

One other reason to study mating behavior is to understand the evolution of "love" darts. Despite the attention that greets each advance, little is known about the use of darts outside of *Cantareus aspersus* (*Helix aspersa* Müller, 1774) (Koene and Chase 1998a, 1998b, Landolfa *et al.* 2001, Rogers and Chase 2001, Rogers and Chase 2002, Koene and Schulenberg 2005, Chase 2006), except that there is considerable variation in the timing of the use of the dart, its morphology, and the number used in different species (Ashford 1883, Tompa 1980, Baminger *et al.* 2000, Koene and Schulenberg 2005). At the extreme of the spectrum, some species of *Euhadra* Pilsbry, 1890 repeatedly stab a dart (~3000 times) during "foreplay" prior to mating (J. Koene and S. Chiba, personal communication). Opinions also vary over what constitutes a dart. Although some might contend that an "amatorial" organ is a dart, we argue that while darts and amatorial organs may (or may not) have similar functions, they are clearly distinguishable because only the former is a "hard, calcified or chitinous organ that is used to pierce a partner during mating" (Davison *et al.* 2005).

There have been very few formal observations of dart use. The only photographs that we are aware of showing darts "in use" are of *C. aspersus* (Koene and Chase 1998a, 1998b, Landolfa *et al.* 2001, Rogers and Chase 2001, Rogers and Chase 2002, Koene and Schulenberg 2005), *Cepaea nemoralis* (Davison *et al.* 2005), and *Trichotoxon heynemanni* (Schilthuizen 2005). It would therefore be useful if creating a database also stimulated malacologists to record dart use and to publicize their efforts. We have recently used information in the database to show that dart-bearing species are confined to the same three monophyletic groups mentioned above (the Helicoidea, Limacoidea, and Philomycidae) and that they all mate face-to-face and simultaneous reciprocally. However, there is no evidence that the relationship is causal (Davison *et al.* 2005).

Although there are still some large clades of snails for which there have been few observations of mating behavior, an interesting dichotomy has emerged between invariant mating position and other highly variable reproductive characters. Some species have head warts (Binder 1977, Takeda 1982, Falkner 1993), penial stimulators (Reise 2004), or amatorial organs (Panha 1987), whereas individuals in other species bite off the penis of their partner (Leonard *et al.* 2002) or entwine their penes before exchanging sperm externally (Quick 1960). Although it has been known that some families tend to have the same shell shape (Cain 1977), the strong and (almost) invariant correlation between mating position and shell shape has mostly been overlooked (Asami *et al.* 1998, Davison *et al.* 2005).

Our attention is drawn to the exceptions. In the helicoid group, species of *Amphidromus* Albers, 1850 have high-spired shells but still mate reciprocally, even between chirally-reversed individuals (M. Schilthuizen, personal communication). As they also lack darts, it is tempting to speculate that the shell-shape change and lack of darts are in some way associated, but there is no firm evidence. Clausiild snails are interesting because some species mate unilaterally whereas others mate reciprocally; there is even within-species variation (Nordsieck 2005a, 2005b). Finally, *Oreohe-*

Table 1. An overview of the literature on the mating behavior of stylommatophoran land snails and slugs. Mating behaviour: FF, face-to-face; I, idiosyncratic; SM, shell-mounting; SR, simultaneous reciprocal; U, unilateral (includes sequential unilateral mating); ?, not known. Shell shape: H, high-spired; L, low-spired; S, slug or semi-slug. Darts: N, dart and art-sac absent; Y, dart or dart-sac present.

Family	Genus	Mating		Shell shape	Darts	References
Helicoidea						
Bradybaenidae	*Bradybaena* Beck, 1837	FF	SR	L	Y	Asami *et al.* 1998
	Euhadra Pilsbry, 1890	FF	SR	L	Y	Takeda and Tsuruoka 1979, Azuma 1995, Asami *et al.* 1998, S. Chiba, A. Davison, J. Koene, pers. obs.
Camaenidae	*Mandarina* Pilsbry, 1895	FF	SR	L	N	S. Chiba & A. Davison, pers. obs.
	Caracolus Montfort, 1810	FF	SR	L	N	Howell-Rivero 1950, Webb 1970b, 1974
	Polydontes Montfort, 1810	?	?	L	N	Webb 1970b, 1974
	Pleurodonte Fischer von Waldheim, 1807	FF	SR	L	N	Sánchez Muñoz 2005a
	Satsuma A. Adams, 1868	FF	SR	L	N	Abbott 1989, Azuma 1995
	Amphidromus Albers, 1850	FF	SR	H	N	Schilthuizen and Davison 2005, M. Schilthuizen, pers. comm.
	Zachrysia Pilsbry, 1894	FF	SR	L	N	Howell-Rivero 1946, Sánchez Muñoz 2005b
Helicidae	*Cepaea* Held, 1837	FF	SR	L	Y	Beaumont 1988
	Cantareus Risso, 1826	FF	SR	L	Y	Giusti and Lepri 1980-1981, Adamo and Chase 1988, Giusti and Andreini 1988
	Theba Risso, 1826	FF	SR	L	Y	Giusti and Andreini 1988
	Arianta Leach in Turton, 1831	FF	SR	L	Y	Hofmann 1923, Locher and Baur 2000
	Helix Linnaeus, 1758	FF	SR	L	Y	Meisenheimer 1907, Jeppesen 1976, Lind 1976, Giusti and Lepri 1980-1981, Chung 1987
	Tacheocampylaea Pfeiffer, 1877	FF	SR	L	Y	Giusti and Lepri 1980-1981
	Eobania P. Hesse, 1913	FF	SR	L	Y	Giusti and Lepri 1980-1981
	Iberus Montfort, 1810	FF	SR	L	Y	Rabaneda-Bueno *et al.* 2004, this paper
Helminthoglyptidae	*Cepolis* Montfort, 1810	FF	SR	L	Y	Webb 1942, 1952a
	Helminthoglypta Ancey, 1887	FF	SR	L	Y	Webb 1942, 1952a, van der Laan 1971, van der Laan 1980
	Humboldtiana Ihering, 1892	FF	SR	L	Y	Webb 1980b
	Monadenia Pilsbry, 1895	FF	SR	L	Y	Webb 1952b
	Sonorella Pilsbry, 1900	FF	SR	L		Webb 1980a, 1990
	Polymita Beck, 1837	FF	SR	L	Y	Moreno 1950, Tur *et al.* 2002
Hygromiidae	*Cochlicella* Férussac, 1820	FF	SR	H	Y	Schileyko and Menkhorst 1997, Asami *et al.* 1998
	Monacha Fitzinger, 1833	FF	SR	L	Y	Storey 2005
	Halolimnohelix Germain, 1913	FF	SR	L	Y	Block 1968a
Polygyridae[2]	*Allogona* Pilsbry, 1939	FF	SR	L	N	Webb 1948a, Emberton 1994, Atkinson 2005
	Ashmunella Pilsbry & Cockerell, 1899	FF	SR	L	N	Webb 1954a, Emberton 1994
	Cryptomastix Pilsbry, 1939	FF	SR	L	N	Webb 1970c, Emberton 1994
	Mesodon Rafinesque in Férussac, 1821	FF[1]	SR	L	N	Webb 1954b, Emberton 1991
	Neohelix Ihering, 1892	FF	SR	L	N	Webb 1952d, Emberton 1994
	Polygyra Say, 1818	FF[1]	SR	L	N	Archer 1933, Emberton 1994, Webb 1994a, 1994b

Table 1. Continued

Family	Genus	Mating		Shell shape	Darts	References
	Stenotrema Rafinesque, 1819	FF[1]	SR	L	N	Webb 1947, 1948b, Emberton 1994
	Trilobopsis Pilsbry, 1939	FF	SR	L	N	Webb 1965, Emberton 1994
	Triodopsis Rafinesque, 1819	FF	SR	L	N	Webb 1948a, 1959, Emberton 1994
	Vespericola Pilsbry, 1939	FF	SR	L	N	Webb 1970a, Emberton 1994
Limacoidea						
Agriolimacidae	*Deroceras* Rafinesque, 1820	FF	SR	S	N	Reise 1995, Reise 2004
Arionidae	*Arion* Férussac, 1819	FF	SR	S	N	Adams 1910, Quick 1946, Davis 1977
	Geomalacus Allman, 1842	FF	?	S	N	Platts and Speight 1988
	Ariolimax Mörch, 1859	FF	SR/U	S	N	Leonard *et al.* 2002
Ariophantidae	*Ariophanta* Desmoulins, 1829	FF	SR	L	Y	Dasen 1933
	Hemiplecta Albers, 1850	FF	SR	L	N	S. Panha, pers. comm.
	Macrochlamys (*Syama*) Godwin-Austen, 1908	FF	SR	L	N	S. Panha, pers. comm.
	Microparmarion Simroth, 1893	FF	?	S	N	Liew Thor Seng, pers. comm.
Gastrodontidae	*Oxychilus* Fitzinger, 1833	FF	SR	L	N	Rodriguez and Gomez 1999
Limacidae	*Limax* Linnaeus, 1758	I[1]	SR	S	N	Chase 1952, Quick 1960, Langlois 1965, Baur 1998
	Limacus Lehmann, 1864	FF	SR	S	N	Barker 1999
Milacidae	*Tandonia* (*Milax*) Lesson & Pollonera, 1882	FF	SR	S	N	Quick 1960
Trochomorphidae	*Bertia* Ancey, 1887	FF	SR?	L	N	Menno Schilthuizen, pers. comm.
Urocyclidae	*Trichotoxon* Simroth, 1889	FF	SR	S	Y	Bernard Verdcourt, pers. comm.
	Gymnarion Pilsbry, 1919	FF	SR	L	N	Binder 1977
	Sheldonia Ancey, 1887	FF	SR	L	N	Herbert and Kilburn 2004
	Elisolimax Cockerell, 1893	FF	SR	S	N	Herbert and Kilburn 2004
Vitrinidae	*Semilimax* Agassiz, 1845	FF	SR	S	Y	Künkel 1933
	Vitrinobrachium Kunkel, 1929	FF	SR	S	N	Künkel 1933
Zonitidae	*Mesomphix* Rafinesque, 1819	FF	SR	L	N	Webb 1952c
	Ventridens Binney & Bland, 1869	FF	SR	L	Y	Pilsbry 1946, Webb 1948c
Other						
Acavidae	*Helicophanta* Westerlund, 1886	SM	U	L	N	George Williams, pers. comm.
Achatinidae	*Achatina* Lamarck, 1799	SM	SR	H	N	Tomiyama 1994, 1996
	Archachatina Albers, 1850	SM	SR	H	N	Plummer 1975
Cerastidae	*Zebrinops* Thiele, 1931	FF	SR	H	N	Block 1968b
Ceriidae	*Cerion* Röding, 1798	?	U	H	N	Woodruff 1978
Chondrinidae	*Solatopupa* Pilsbry, 1917	?	U	H	N	Boato and Rasotto 1987
Clausiliidae	*Albinaria* Vest, 1867	SM	SR	H	N	Schilthuizen and Lombaerts 1995, Menno, Schilthuizen, pers. comm.
	Euphaedusa O. Boettger, 1877	SM	U	H	N	Asami *et al.* 1998
	Luchuphaedusa Pilsbry, 1901	SM	U	H	N	Asami *et al.* 1998
	Stereophaedusa O. Boettger, 1877	SM	U	H	N	Asami *et al.* 1998
(Alopilinae)	*Agathylla* H. & A. Adams, 1855	SM	SR	H	N	Nordsieck 1969, 2005a, 2005b
	Cochlodina Férussac, 1821	SM	U	H	N	Nordsieck 2005a, 2005b
	Delima Hartmann, 1842	SM	SR	H	N	Nordsieck 1969, 2005a, 2005b
	Herilla H. & A. Adams, 1855	SM	SR	H	N	Nordsieck 2005a, 2005b
	Medora H. & A. Adams, 1855	SM	SR	H	N	Nordsieck 2005a, 2005b
(Baleinae)	*Balea* Gray, 1824	SM	U	H	N	Nordsieck 2005a, 2005b
(Clausiliinae)	*Laciniaria* Hartmann, 1842	SM	U	H	N	Nordsieck 2005a, 2005b
	Macrogastra Hartmann, 1841	SM	U	H	N	Nordsieck 2005a, 2005b
	Clausilia Draparnaud, 1805	SM	U	H	N	Nordsieck 2005a, 2005b
	Ruthenica Lindholm, 1924	SM	U	H	N	Nordsieck 2005a, 2005b
	Neostyriaca A. Wagner, 1920	SM	U	H	N	Nordsieck 2005a, 2005b

Table 1. Continued

Family	Genus	Mating		Shell shape	Darts	References
Discidae	*Anguispira* Morse, 1864	SM[1]	SR	L	N	Webb 1968b
Enidae	*Mastus* Beck, 1837	SM	SR	H	N	Paramakelis and Mylonas 2002, Paramakelis, pers. comm.
Haplotrematidae	*Haplotrema* Ancey, 1881	SM	U	L	N	Webb 1943
Oreohelicidae	*Oreohelix* Pilsbry, 1904	SM	U	L	N	Webb 1951
Orthalicidae	*Liguus* Montfort, 1810	SM	U	H	N	Davidson 1965, Poland 2005
Partulidae	*Partula* Férussac, 1819	SM	U	H	N	Lipton and Murray 1979
Philomycidae	*Philomycus* Rafinesque, 1820	FF	SR	S	Y	Webb 1968a
Rhytididae	*Paryphanta* Albers, 1850	SM	U	L	N	Stringer *et al.* 2003
Spiraxidae	*Euglandina* Fischer & Crosse, 1870	SM	U	H	N	Cook 1985
Strophocheilidae	*Strophocheilus* Spix, 1827	SM	U	H	N	Wiswell and Browning 1967
Succineidae	*Catinella* Pease, 1870	SM[1]	SR	H	N	Webb 1977a
	Oxyloma Westerlund, 1885	SM	U/SR	H	N	Webb 1977a, 1977b, 1977c
	Succinea Draparnaud, 1801	SM	U/SR	H	N	Rieper 1912, Hecker 1965, Webb 1977a, Jackiewicz 1980, Villalobos *et al.* 1995, Jordaens *et al.* 2005
Valloniidae	*Vallonia* Risso, 1826	FF	SR?	L	N	Barker 1999
Vertiginidae	*Vertigo* Müller, 1774	SM	U?	H	N	Barker 1999

[1] External sperm exchange; sperm is deposited on the male's everted penis without intromission (see Emberton 1994)

[2] See Emberton (1994) for details of mating in other Polygyrid species (mostly papers by Webb).

Table 2. Species for which there are videos showing mating behavior.

Taxon	Mating behavior	Film-maker
Ariolimax dolichophallus Mead, 1943	Face-to-face	Brooke Miller, UC Santa Cruz (miller@biology.ucsc.edu) http://bio.research.ucsc.edu/grad/weaver/Pages/project.html
Cantareus aspersus Müller, 1774	Face-to-face	Joris Koene, Vrije Universiteit (joris.koene@falw.vu.nl) Ronald Chase, McGill University (ronald.chase@mcgill.ca)
Deroceras sp.	Face-to-face	Heike Reise, Staatliches Museum fur Naturkunde Gorlitz (Heike.Reise@smng.smwk.sachsen.de http://www.malacsoc.org.uk/Malacological%20Bulletin/BULL43/king2.htm#dive
Euhadra sandai Kobelt, 1879	Face-to-face	Nishi Hirotaka (movie archives of animal behaviour) http://zoo2.zool.kyoto-u.ac.jp/ethol/
Satsuma amanoi Kuroda, 1960	Face-to-face	Nishi Hirotaka (movie archives of animal behaviour) http://zoo2.zool.kyoto-u.ac.jp/ethol/
Mastus pupa Linnaeus, 1758	Shell-mounting	Aris Paramakelis, University of Crete (parmakel@nhmc.uoc.gr)

lix Pilsbry, 1904 is another intriguing genus because it mates by shell-mounting (Webb 1951). Although its phylogeny is unclear (Wade *et al.* 2006), we predict that it will either fall within the helicoid group or be basal to it.

We expect that observations of mating behavior will grow over the coming years and so will continue to update the database of mating behavior (updated versions at http://www.molluscs.org). We welcome any data that we have inadvertently excluded, including single photographs.

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The function of dart shooting in helicid snails*

Ronald Chase

Department of Biology, McGill University, 1205 Ave. Docteur Penfield, Montréal, Québec, H3A 1B1 Canada, ronald.chase@mcgill.ca

Abstract: Some stylommatophoran species, including several helicid snails common to Europe and North America, drive sharp, calcareous darts into their sexual partners prior to copulation. Why any animal would treat a prospective mate in this manner has been the subject of considerable speculation. One widely held belief is that the dart stimulates the partner. Here, I review evidence showing that this hypothesis, along with several others, is almost certainly incorrect. On the other hand, there is strong empirical support for the idea that the dart increases the reproductive fitness of the successful shooter by promoting the survival and utilization of its sperm. How the dart works to produce this effect is an open question; current evidence indicates that it injects a chemical agent into the recipient and that this substance contracts the female tract in such a manner as to facilitate the passage of allosperm to the spermatheca. Although successful dart shooting clearly benefits the shooter, there is little evidence to suggest either a cost or a benefit to the recipient.

Key words: sexual selection, sperm competition, courtship, sexual conflict, *Cantareus aspersus*

According to recent phylogenetic studies (Koene and Schulenburg 2005, Davison *et al.* 2006), a dart is used in only 4-9 families within the Stylommatophora, which comprises approx. 60 families of snails and slugs in total. It is noteworthy that all the dart-bearing families mate in a simultaneous, reciprocal manner (Davison *et al.* 2006). Most of the research on molluscan darts has been done on helicid snails, in particular *Cantareus aspersus* (Müller, 1774; formerly *Helix aspersa*). Therefore, the present review relates specifically to *C. aspersus* unless otherwise noted.

The dart, like other reproductive structures, differs greatly in size and shape among species (Koene and Schulenburg 2005). Individuals of a few species even possess multiple darts, all of which are released in a single courtship episode. In *Cantareus aspersus*, the dart is sharply pointed; it has a fluted shaft and a corona by which it attaches to the dart sac while in storage (Fig. 1). The dart from the first shooter is released about 30 min before the initiation of courtship (Fig. 2). About 25 min later, the second animal releases its dart. Copulation (mutual intromission) ensues after a further 10 min (Chung 1987, Adamo and Chase 1988). Although the act of releasing the dart is often described as "shooting," in fact the dart does not travel through the air. It is forcefully externalized, but its corona remains lightly attached to the dart sac until it is pulled away after the tip becomes embedded in the partner's skin. Approximately one-half of the darts strike the body wall of the partner and remain lodged there for hours, whereas the rest of the darts either miss the intended target altogether or strike only weakly, then fall out. In the former cases, the dart is retracted. Significantly, copulation occurs regardless of the fate of either partner's dart.

From appearances, it would seem that the dart is harmful, but this has not been proven. Although I have observed hundreds of matings, I have never seen any reaction to the dart apart from a momentary reflexive withdrawal of the body. Never has an animal suffered a noticeable long-term effect, let alone death. However, I have seen one dart penetrate cleanly through the head of its target (Fig. 1 in Chase and Blanchard 2006) and another dart lodge in the cerebral ganglion.

The possibility of interactions between the two dart shooting events of a courtship was examined in a recent study (Chase and Vaga 2006). We found that neither the timing, accuracy, nor location of the second shot was influenced by the success or failure of the first shot. This result, and others, indicates that dart shooting is not a source of conflict during the mating process: the protracted courtships cannot be interpreted as attempts to shoot without being shot. Rather, each snail appears to be interested only in getting off the best possible shot, evidently one that penetrates deeply near the genital pore, for reasons to be explained below. Although we found no evidence for direct costs of dart receipt, the possibility of indirect, post-copulatory costs remains a possibility; this too will be discussed below.

FOUR FALSIFIED HYPOTHESES

The striking behavior of dart shooting has occasioned numerous commentaries through the ages, with no paucity

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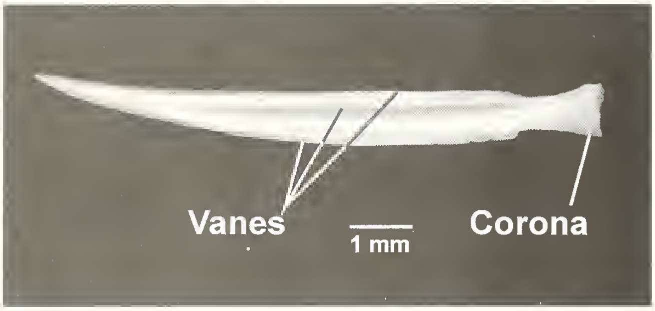

Figure 1. Photograph of a dart from *Cantareus aspersus*. The corona remains attached to the tubercle of the dart sac until the dart strikes its target. As the dart is expelled, mucus collects in the fluted cavities created by the vanes.

of speculation as to its function. Here, I briefly review several hypotheses which, although once plausible, may now be rejected (see also Landolfa 2002).

1. Sexual stimulation

Swammerdamm described the dart in the mid seventeenth century, but he was apparently unaware of its role in reproduction (for references, see Kothbauer 1988). The first written account of dart shooting is by Maupertuis (1753). Maupertuis' interpretation of the dart's function was precisely that of most modern authors, namely that it stimulates the partner to proceed with mating. Kothbauer (1988) has drawn our attention to the fact that Maupertuis' view of dart shooting in snails, as well as that of many subsequent authors, corresponds to the main idea behind Eros, the Greek god who was able to cause other gods to fall in love by shooting them with arrows. Indeed, although I have no evidence, I suspect that the ancient Greeks created Eros after observing *Cantareus aspersus* (or perhaps *Helix pomatia* Linnaeus, 1758) shooting darts in their gardens. Maupertuis asserted that the snail's use of the dart is necessary and justifiable due to the snails' lethargic disposition, but he argued that for humans to use similar violent means to arouse passions would be immoral.

An immediate objection to the idea that the dart's function is to stimulate the partner is that by the time the dart is shot, *i.e.*, late in the courtship ritual, the partner is already highly aroused; indeed the partner is nearly ready to shoot its own dart. Hence, at this point, there is no need to further stimulate the partner.

Several empirical studies have examined whether the receipt of a dart does, in fact, quicken the activity of the targeted partner. Adamo and Chase (1988) found that the interval between the two dart shots was slightly reduced when the first shot hit the partner compared to when it missed the partner; these data suggested a stimulatory effect of the dart. However, other studies (*e.g.*, Lind 1976, *Helix pomatia*) reported either an absence of stimulation or an actual diminution in the level of arousal after a snail was hit by a dart. Additionally, in a recent study with a large sample size and strictly defined measures, we found no significant effect of successful dart shooting on the interval between dart shots (Chase and Vaga 2006). Nor did we find that the outcome of either dart shot affected the duration of courtship (measured from the first dart shot to intromission).

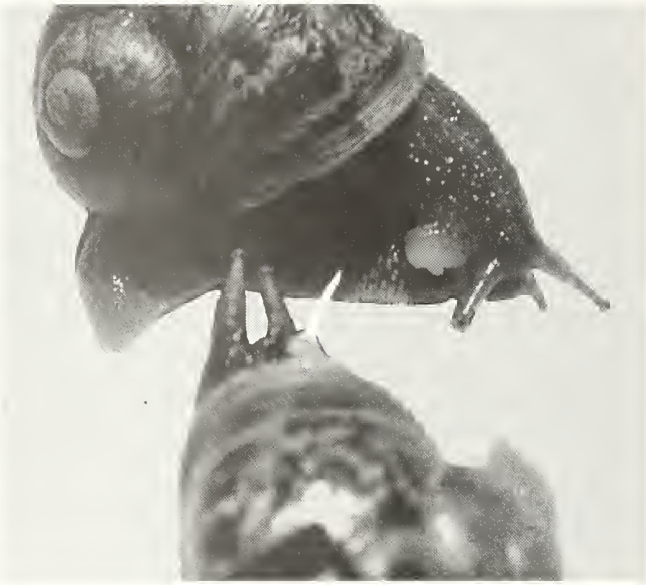

Figure 2. An individual of *Cantareus aspersus* photographed at the moment of dart release. Because this dart did not penetrate the partner, it was retracted by the shooter and digested. The peculiar squeezed appearance of the shooter's tentacles is the consequence of elevated hydrostatic pressure. Note also the everted genital apparatus of the intended target snail. The photograph was digitally edited to eliminate background and to enhance contrast; the dart was colored white. Shell length of the upper snail is ca. 27 mm. Original photograph by Shelley Adamo; photograph reproduced with permission of Oxford University Press.

2. Species recognition

This hypothesis (Diver 1940) is built on the fact that snails lack an auditory sense and have essentially no vision, leaving only touch and chemosensation as instruments by which to distinguish conspecifics from heterospecifics. The hypothesis is effectively disproven by the fact that snails show no reluctance to mate even when untouched by their partner's dart. An alternative, and likely, means of identifying conspecifics is through the extensive body contacts that occur during courtship.

3. A gift of calcium

Charnov (1979) proposed that the dart is a gift of calcium that can be used to promote the development of offspring. It is true, of course, that young snails require an ample supply of calcium to grow their shells, and that environmental sources of the mineral may be limited. On logical grounds, however, it would seem to be a poor strategy to give away as much calcium by shooting a dart as one is likely to get by receiving a dart. In any case, the amount of calcium that can be effectively transmitted to the offspring from a donated dart is too small to make an appreciable difference (Koene and Chase 1998a).

4. A signal of intention

In an attempt to solve the enigma of the dart, Leonard (1992) advanced the idea that snails shoot darts to signal their readiness to deliver sperm to their partner. This hypothesis grew out of earlier work in which she claimed that the male role is the less preferred role in the helicid mating system because the fate of donated sperm is uncertain. Thus, she argued, snails would rather mate as females. The evolution of the dart provided an honest signal of a snail's intention to donate sperm, thus inducing the partner to reciprocate and allowing both snails to benefit. Leonard's bold hypothesis, however, has been contested on both theoretical and empirical grounds. First, the assumed preference for the female role is untenable because, over time, the fitness of male actors and female actors is exactly equal (Greeff and Michiels 1998). Second, several of Leonard's specific predictions have been falsified (Adamo and Chase 1996). Critically, snails that do not receive darts nevertheless intromit and they deliver full spermatophores to their non-shooting or poorly shooting partners (Rogers and Chase 2001, Chase and Vaga 2006).

ONE SUPPORTED HYPOTHESIS

The only hypothesis to receive consistent empirical support states that successful dart shooting enhances male fitness by allowing more of the shooter's sperm to become stored in the recipient's spermathecal sacs, hereafter referred to as the sperm-loading hypothesis. As first proposed by Chung (1987) and later elaborated upon by Adamo and Chase (1996), the sperm-loading hypothesis treats dart shooting as a male manipulative device while ignoring female interests, but I discuss the female point of view below. In addition, neither Chung (1987) nor Adamo and Chase (1996) explicitly referred to the concept of sperm competition, *i.e.*, competition between males to fertilize eggs, although it is in this context that the hypothesis is correctly placed today. Helicid snails are ideal participants for sperm competitions because they mate promiscuously, they store sperm for long periods of time, and they fertilize internally (Chase 2002).

In helicid snails, sperm are packaged inside a spermatophore for transfer during copulation. After the spermatophore is delivered to the partner, the sperm leave the spermatophore and migrate to the storage site, a structure known as the fertilization pouch–spermathecal complex (FPSC). Along the way, digestive enzymes typically digest about 99.98% of the received allosperm (Lind 1973, Rogers and Chase 2001).

Strong evidence in favor of the sperm-loading hypothesis came from the study of Rogers and Chase (2001). Virgin snails were mated one time only with partners that either hit them with their darts or missed them with their darts. Seven days after the mating, the former virgin was dissected and the FPSC was removed. Allosperm in the FPSC were labeled using a fluorescent DNA stain, then counted. Snails that were hit by a dart stored 116% more sperm than snails that were missed (Rogers and Chase 2001). Because helicid snails can produce multiple egg clutches from the sperm of a single donor (Chen and Baur 1993), the results of this experiment imply a fitness advantage to the successful dart shooter because its sperm should remain available for a larger number of clutches than would be the case if its dart had missed.

To see whether the increased sperm storage that we observed after a single mating would provide an advantage when the successful shooter competed with a second sperm donor, we conducted competitive mating trials in which one donor hit the recipient with his dart and the other donor missed. The order of hits and misses was balanced. After the matings, we waited for eggs to be laid and then genotyped the twice-mated mother, each of the two potential fathers, and a randomly chosen sample of the offspring. Note that if the sperm used for fertilization were selected by a raffle-like process, then the donor that has managed to store the most sperm will be the most successful father. Thus, we predicted that the successful dart shooter would father more offspring than the unsuccessful shooter. The experiment was conducted twice, with slightly different conditions, but with very similar results (Landolfa *et al.* 2001, Rogers and Chase 2002). Successful dart shooting significantly improved paternity in this competitive mating situation (Fig. 3). In *Cantareus aspersus*, sperm from the first donor is used preferentially over that from the second donor, regardless of the success or failure of dart shooting (Evanno *et al.* 2005, Chase and Blanchard 2006). As a consequence of this phenomenon, known as first donor precedence, the influence of the dart is most pronounced with respect to the second donor. The paternity of the second donor increased from 17%, when its dart missed and the first donor's dart hit, to 39% when its dart hit

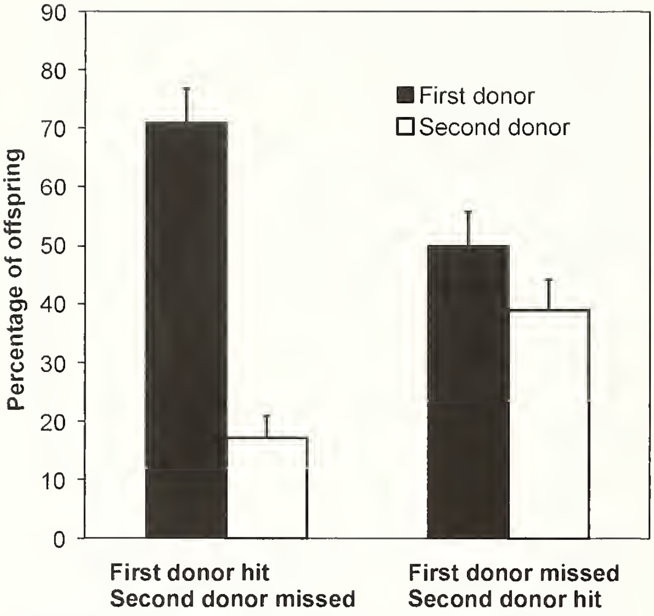

Figure 3. Successful dart shooting increases reproductive success in competitive matings. Snails mated with two sperm donors before producing offspring. One donor hit with its dart, the other missed. Paternities were determined by allozyme genotyping; means ± SE are shown. The effects of the dart are most evident with respect to the second donor (17% paternity *vs.* 39% paternity), owing to first sperm precedence. Data are from Rogers and Chase (2002).

and the first donor's dart missed (Fig. 3; Rogers and Chase 2002).

UNRESOLVED QUESTIONS

While considerable progress has been made in understanding the function of the dart, several questions remain concerning its mechanism of action and its evolution.

1. How does the dart influence sperm utilization?

We suppose that receipt of a dart triggers events in a signaling pathway that ultimately produces effects in the organs that receive sperm. A complete account of the dart's mechanism of action will require descriptions of each step in the signaling pathway. Here, I focus on the initial signal, which is conveyed by the dart. Although the dart itself is a hard structure that will elicit responses in mechanosensory neurons when it penetrates the body wall, the possibility that the dart carries a chemical signal must also be considered because the dart is covered with mucus when it is expelled by the shooter. The presence of mucous glands specifically associated with the dart sac is a consistent feature of the dart-bearing stylommatophoran species (Koene and Schulenburg 2005). The mucus produced by these glands could be used simply to lubricate the passage of the dart out of the animal. However, several pieces of evidence suggest that it contains one or more chemical components that are essential effectors of the dart's function. First, the fluted structure of the dart itself (Fig. 1) can be seen as an adaptation to increase the amount of mucus that can be loaded onto the dart. In *Cantareus aspersus*, the dart carries about 2 mg of mucus (Chung 1986). Second, quantitative analysis has revealed that the effect of dart shooting on sperm storage and paternity are both significantly dependent on the shell volume of the recipient (Rogers and Chase 2001, 2002). The smaller the recipient snail, the larger the dart's effect. One interpretation of this relationship is that the potency of the dart is diminished in larger animals due to chemical dilution. Third, when mucus from the dart gland is applied to the female reproductive tract *in vitro*, contractions occur that cause a reconfiguration of the tract at the critical junction between the organ that receives the spermatophore (the bursa tract diverticulum) and the sperm digestive gland (the bursa copulatrix) (Koene and Chase 1998b). These mucus-induced contractions could allow more sperm to escape enzymatic digestion as they travel from the safety of the spermatophore to the safety of the spermatheca.

Based on the observations summarized above, it is reasonable to suppose that the dart functions by injecting mucus into the recipient and that molecules present in the mucus cause temporary structural changes in the female tract. According to this idea, the dart serves only to convey and inject the chemical agent. To test the hypothesis, Katrina Blanchard and I conducted an experiment to determine whether the dart works by a mechanical means or a chemical means (Chase and Blanchard 2006). As in the experiments described above (Landolfa *et al.* 2001, Rogers and Chase 2002), we arranged for a snail to receive sperm from two donors before producing offspring. In this experiment, however, none of the snails shot darts. Instead, we poked the eventual mother with a hypodermic needle as soon as we detected the partner's intention to dart-shoot. In one of the two matings, we injected saline through the needle; in the other mating, we injected an extract of the dart gland mucus. Thus, in both cases, the mother received mechanical stimulation, but only in the latter case did she receive chemical stimulation. On average, snails delivering sperm in association with injections of mucus fathered 2.3 times the number of babies as did competing snails that delivered sperm to the same mother in association with injections of saline (Chase and Blanchard 2006). This result provides strong evidence that the dart works largely or entirely by injecting mucus, not simply by rupturing the skin. We cannot exclude the possibility, however, that skin rupture alone may have a small effect on paternity.

2. What is the identity of the bioactive molecule(s) in the dart's mucus?

The next step will be to identify the bioactive molecule(s) in the dart's mucus. Because gastropod molluscs often use peptides as neurotransmitters and hormones (Chase 2002), and because peptides have been found in the secretions of the mucous gland (Börnchen 1967, Chung 1986), the effective agent is likely to belong to this class of molecule. To identify the molecule requires an efficient bioassay. At the present time, a modified procedure to count stored allosperm in once-mated individuals offers the best opportunity. By adopting an approach based on the successive fractionation of mucous extracts, and by using sperm counts as the bioassay, it should be possible to identify the molecule(s) of interest.

3. Does dart shooting either benefit or harm the recipient?

While successful dart shooting almost certainly benefits the shooter, it is not known whether it either benefits or harms the recipient. Benefits to the recipient would occur in either of two scenarios: (1) if successful dart shooting were associated with genes that provide superior viability (the "good genes" model) or (2) if the ability to shoot successfully were heritable, in which case the offspring of the recipient would have a fitness advantage (the "sexy sons" model). Although there is as yet no evidence bearing on these possibilities, suitable tests could be conducted. To test the "good genes" model, it will be necessary to compare the longevity, or viability, of successful and unsuccessful shooters. To test the "sexy sons" model, it needs to be shown that variability in dart structure, mucous content, or dart-shooting behavior is heritable. Until one of these tests provides positive evidence of a benefit to the recipient (and none will be easy to perform), it is not unreasonable to assume a benefit to the shooter alone, in which case dart shooting could be characterized as "male manipulation" (Adamo and Chase 1996).

Rather than benefiting the recipient, the dart could be costly if it reduced the recipient's control over the process of fertilization, if it damaged tissue, or if it increased the chances of infection via the wound. However, none of these possible long-term effects has been documented, and short-term negative effects appear negligible (Chase and Vaga 2006). Thus, current evidence indicates that while successful dart shooting benefits the shooter, its consequences for the recipient are neutral.

Bearing in mind that the recipient is the female partner in this drama, and assuming for the moment that successful dart shooting is associated with high quality genes, it has been proposed (Landolfa 2002) that females perceive the successful dart shot as an indicator of good genes and that they therefore "choose" to store and use sperm from the successful shooter. This would amount to mate selection, but with the choice being made after copulation, *i.e.*, in a "cryptic" manner (Eberhard 1996). It is conceivable that the female function could sort the sperm from various donors to different spermathecal sacs, and later, prior to fertilization, she could selectively release the sperm belonging to the highest-quality donor (see Bojat *et al.* 2001). Attractive though this idea may be, there is no evidence to support it. Furthermore, as noted above, we recently found that injections of mucus through a needle can replicate the benefits to male fitness that ordinarily follow from successful dart shooting. Thus, if females are choosing, they could only be doing so on the basis of the mucus. The use of any other trait, including any present in the shooting behavior, the dart structure, or the sperm, is excluded by the design of the aforementioned experiment.

4. How did the dart evolve?

The steps in the evolution of the dart apparatus are difficult to imagine and probably impossible to confirm. There are many types of "accessory" or "auxiliary" structures associated with the stylommatophoran penis (Tompa 1984). These structures comprise two major groups: (1) the sarcobelum, a fleshy club-like appendage, and (2) the gypsobelum, a hard, sharp instrument, of which the dart is just one example. Although glands are certainly associated with accessory structures in many species, it would be useful to learn the full extent of this association. If glands were invariably associated with accessory structures, then this would support my contention that the primary adaptation is the evolution of a bioactive agent capable of influencing paternity. In ancestral cases, the substance might have been secreted, unaided, out the genital pore. Subsequently, different lineages may have independently evolved accessory structures to improve the efficiency of delivery of the secretion product. Alternatively, if there were taxa that possess either a sarcobelum or a gypsobelum but no gland, then perhaps the accessory structure itself is able to enhance paternity. As mentioned earlier, our experimental evidence in *Cantareus aspersus* is insufficient to rule out this possibility (Chase and Blanchard 2006). In this latter scenario, the glandular product would be a secondary development that increased the power of the manipulation.

If, in fact, the recipient of a dart suffers a cost to its female function, then an evolutionary arms race (Parker 1979) may evolve in which adaptations are selected that on the one hand maximize the dart's efficacy and on the other hand minimize the extent of the harm caused by it. Koene and Schulenburg (2005) recently reported results that are consistent with this picture. From a phylogenetic analysis, they found an association between a multi-component measure of the dart's shape and a multi-component measure of the "complexity" of the bursa tract diverticulum. Species that have small darts have short diverticula, whereas species with large darts or highly curved darts have long diverticula. This result can be interpreted in light of the fact that longer diverticula make it more difficult for sperm to escape safely (Lind 1973). Thus, it would appear that, as species evolved, the female function selected longer diverticula to defend itself from the harmful effects of more powerful darts. However, until a specific cost of dart receipt is documented, questions relating to sexual conflict and its attendant antagonistic coevolution with respect to the dart will remain controversial.

CONCLUSION

Substantial progress has been made in recent years on the question of why snails shoot darts. In *Cantareus aspersus*, the dart is used to increase the survival and storage of the shooter's sperm in the recipient's spermathecal sacs. As a consequence, successful shooters have greater reproductive success. The phenomenon can be characterized as one of post-copulatory sexual selection in the context of intense sperm competition. To describe it in such terms would have been impossible prior to the bloom of sexual selection theory in the mid-twentieth century, thus explaining, I believe, why it took so long for the riddle of the dart to be solved. Not until empirical work on birds and insects had made known the details of post-copulatory sexual selection in those animals, and theoreticians had elaborated a general context able to accommodate other taxa, could one have imagined the dart's hidden function. Studies on species other than *C. aspersus* are needed to generalize the findings that are summarized in this paper and to provide insights into the dart's evolution.

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Accepted: 1 July 2006

MEETING ANNOUNCEMENT

74th Annual Meeting of the American Malacological Society

Carbondale, Illinois

The American Malacological Society will hold its 74th annual meeting in Carbondale, Illinois from June 29–July 3, 2008. The venue will be the Southern Illinois University Student Center, which houses an auditorium, several ballrooms and meeting rooms, and a number of restaurants and coffee shops. The conference will begin with an icebreaker on Sunday evening. Special events will include an outdoor reception at Blue Sky Vineyard (www.blueskyvineyard.com) on Monday night, a poster session and the AMS Auction of molluscan miscellany on Tuesday night, and a barbecue banquet (with vegetarian options) at the 17th Street Bar & Grill Warehouse, Southern Illinois' most unique banquet facility (www2.murphysboro.com/community/restaurant/17thstreet.html) on Wednesday night. There are persistent rumors of a dance party on Tuesday or Wednesday night as well. The special sessions and symposia will include:

- a land snail conservation symposium and workshop in honor of the late Leslie Hubricht, organized by Kathryn Perez (University of North Carolina–Chapel Hill/Duke University), Jay Cordeiro (NatureServe), Jochen Gerber (Field Museum of Natural History), and Kevin Roe (Iowa State University)
- a symposium on molluscan taxonomy in the 21st century, organized by Benoît Dayrat (UC Merced)
- a special session on cephalopod biology organized by Dr. Frank Anderson, Dr. Christine Huffard (Monterey Bay Aquarium Research Institute), and Dr. Elizabeth Shea (Delaware Museum of Natural History)

On Thursday, two field trips will introduce meeting participants to two wonderful mollusk habitats in southern Illinois. Participants will be able to take a tour of the Larue Pine Hills/Otter Pond Research Natural Area, a fantastic area of limestone bluffs and outcrops (and home of *Euchemotrema hubrichti*, the conference mascot) or a trip to local aquatic habitats to search for freshwater bivalves and gastropods.

Visitors to Carbondale usually travel through either St. Louis or Chicago, though Memphis is also an option. There is a convenient shuttle service from St. Louis Lambert International Airport to Carbondale. From Chicago or Memphis, you can take a train—*The City of New Orleans*, which stops in Carbondale on its Chicago-to-New Orleans route. American Connections regional commuter flights arrive at the Williamson County airport (618-993-3353) several times daily. The airport is located 16 miles east of the SIU campus.

We look forward to seeing you in Carbondale, Illinois in 2008!

Frank E. (Andy) Anderson, President (2008)
American Malacological Society
feander@siu.edu

Amer. Malac. Bull. **23**: 191-194

Melbourne Romaine Carriker: 25 February 1915 – 25 February 2007

An Appreciation

Clement L. Counts, III[1], Robert S. Prezant[2], and J. Evan Ward[3]

[1] Department of Biological Sciences, Richard A. Henson School of Science and Technology, Salisbury University, Salisbury, Maryland 21801-6861, U.S.A., clcounts@salisbury.edu

[2] College of Science and Mathematics, Montclair State University, Montclair, New Jersey 07043, U.S.A., prezantr@mail.montclair.edu

[3] Department of Marine Sciences, University of Connecticut, 1080 Shennecossett Road, Groton, Connecticut 06340, U.S.A., evan.ward@uconn.edu

On 25 February 2007, our mentor, colleague, and great friend, Melbourne Romaine Carriker died at Lewes, Delaware. It was his ninety-second birthday. He was surrounded by his children and grandchildren.

Mel's life was as eventful and full as his scientific career. He was born 25 February 1915 to Melbourne Armstrong Carriker, Jr. and Myrtle Carmella Carriker on the family coffee plantation, *Vista Nieve*, near Santa Marta, Colombia. Mel detailed his boyhood experiences on the plantation in his memoir *Vista Nieve* (Carriker 2000). In 1925, at the age of ten, Mel participated in his first biological expedition accompanying his father, a world-class ornithologist and entomologist, to the eastern slope of the Andes.

The plantation was sold in 1927. After the sale, the family moved to Tom's River, New Jersey, and Mel's father became a curator of birds at the Academy of Natural Sciences of Philadelphia (ANSP). Mel attended the public schools and graduated from high school in 1934. In 1934 and early 1935, Mel and his father returned to the Andes in Bolivia on another ornithological expedition (Carriker, Jr. 2006). During the steamship trip, Mel demonstrated his remarkable abilities on the dance floor, exhibiting such skill that other dancers stopped to watch him and his partner. These displays were attributed to lessons provided by Mel's mother in Tom's River (Castillo and Holyoak 2004). This journey to the Andes was epic with train travel to the Alto Plano, a steamer across Lake Titicaca, and brushes with Bolivian troops fighting a war with Argentina (Carriker 2005, Carriker, Jr. 2006). It was during this expedition that Mel contracted malaria.

Mel entered Rutgers University in 1935 and he majored in agricultural research and minored in zoology, graduating with honors and a B.S. in Zoology in 1938. Mel noted, by playing a few minutes in a varsity match, he lettered in water polo. It was Mel's aim to become an ornithologist but in 1938, his undergraduate advisor, Thurlow C. Nelson, offered him a position to study population movements of oyster larvae in Barnegat Bay, New Jersey. In fall 1938, he entered the University of Wisconsin and there earned a Master of Philosophy and, then, a Doctor of Philosophy degree in June 1943. During summers from 1938 through 1941, Mel returned to Great Bay, New Jersey, and in the summer of 1942 he was placed in charge of the Oyster Investigation Laboratory at Bivalve, New Jersey. These experiences launched his research on Mollusca. Mel joined the graduate student group of Lowell E. Noland to study *Lymnaea stagnalis* (Linné, 1758), the snail vector for swimmer's itch in humans. His doctoral dissertation focused on radular and digestive anatomy, physiology, and function of *L. stagnalis.*

During 1939 at Wisconsin, Mel met Meriel Roosevelt McAllister, known as Scottie. Following graduation from Wisconsin, Mel entered the Naval Officers Training program at Harvard College in June 1943 and emerged an Ensign in the United States Naval Reserve. On 17 October 1943, he and Scottie were married in Richmond, Virginia, at a ceremony officiated by Scottie's uncle. Mel and Scottie would have four sons: Eric, Bruce, Neal, and Robert. Mel was ordered for further training at Fort Schuyler, New York, followed by training in Miami, Florida. Mel was then ordered to the Aleutian Islands to serve aboard a small patrol craft with a crew of 60 men and 5 officers. Since the Japanese had been absent for several months, there was little to do but make patrols, during which his duties were standing watch and burning obsolete codes. Eventually, he was promoted to Lieutenant (junior grade) and was made executive officer (second-in-command). Between patrols, Mel collected muricid gastropods and their blood sera from the Aleutian waters for shipment. Mel laughed that the seamen thought this behavior was odd but forgave him because he was, after all, an officer, so odd behavior was expected. Mel placed these Alaskan collections in the alcohol-preserved collections at ANSP in the mid-1980s. Eventually his ship was sent to Pearl Harbor for escort duty, including escorting barges filled with pineapples. At the war's end, Mel was ordered to report for duty aboard a destroyer, patrolling off the Philippines, and became a civilian again on 25 December 1945.

Mel and his family moved in with his mother at Belmar, New Jersey, though he spent some time at Madison, Wisconsin, publishing his dissertation. Although Mel had five offers for positions, he was persuaded by Thurlow Nelson to return to Rutgers and became a Lecturer of Zoology in 1946. Mel came to regret taking the position since many of the faculty remembered him as an undergraduate and still thought of him as such. Then as an Assistant Professor at Rutgers, he developed a graduate course in estuarine ecology and participated in field courses where students and Mel's colleagues from geology and botany studied one of three transects across the state. During 1947-1951, Mel, Thurlow Nelson, and Harold Haskin conducted studies on *Mercenaria mercenaria* (Linné, 1758) with a view to commercialization. Nelson and Haskin worked in Delaware Bay while Mel worked on Little Egg Harbor, New Jersey. By 1954, it became evident that Rutgers had room for only two marine biologists and Mel opted to accept a position as Assistant Professor at the University of North Carolina at Chapel Hill. During 1954 and 1955, Mel conducted research on oysters and clams on Gardner's Island, New York under the sponsorship of the U.S. Fish and Wildlife Service in cooperation with Victor Loosanoff.

While at UNC, Mel spent 1956 to 1960 doing research at North Carolina Institute of Fisheries Research. He also cooperated at the National Marine Fisheries Service Laboratory at Morehead City and the Duke University Marine Laboratory at Beaufort, North Carolina. During these summers, Mel focused his research on gastropods that drilled oysters. The chair of the department, Charles Jenner, headed both the limnology and marine ecology divisions of the department and undervalued Mel's contributions to the point that Mel was dismissed in 1961.

Mel then accepted a position at the U.S. Fish and Wildlife Service. Mel and his family moved to Easton, Maryland in the fall of 1961 and he took up his position at the Bureau of Commercial Fisheries Laboratory at Oxford, Maryland. As Chief of the Shellfish Mortality Program, Mel was in charge of research on MSX, the parasitic disease of *Crassostrea virginica* Gmelin, 1791 that was gaining a substantial hold on oyster populations in Chesapeake Bay. However, funding was problematic and frustrating. Just as Mel was beginning at Oxford, he was offered the position as director of the newly established Systematics-Ecology Program at the Marine Biological Laboratory at Woods Hole, Massachusetts.

The Systematics-Ecology Program operated successfully between 1962 and 1972 to study the flora and fauna of the western North Atlantic. Mel developed the keys to Woods Hole Region with Ralph I. Smith (Smith 1964). Mel believed that the accurate identification of species was central to good ecological practice. In furtherance of this belief, Mel developed and supervised the publication of the series *Keys to the Flora and Fauna of the Northeast Atlantic Coast* for the National Marine Fisheries Service. During this time, Mel also served on the Northeastern Regional Council, assembled by the American Institute of Biological Sciences to study bioscience research to be conducted on a manned Earth-orbiting space station (Olive and Beem 1967). Mel famously made a motion picture of the drilling behavior of *Urosalpinx cinerea* (Say, 1822) that has since been placed on the Internet (http://www.iwf.de/iwf/do/mkat/details.aspx?Signatur=C+13067), along with amplified recordings of the rasping of the radula of muricids as they drilled through oyster shell. By 1972, federal funding was becoming scarce and Mel accepted a full professorship at the College of Marine Studies of the University of Delaware.

Mel (Fig. 1) was responsible for helping to lay out the new Harry L. Cannon Laboratory. He was instrumental in developing the shellfisheries program, and a new species of ameba found in the tanks was named in his honor (*Ovalopodium carrikeri* Sawyer, 1980). Mel taught graduate courses in malacology and supervised the research of doctoral students (see Carriker on AMS web site: Table 1) and master's students (AMS web site: Table 2). Mel also recruited experts in marine ecology who presented summer graduate courses. Mel served on the doctoral and master's committees of over 150 individuals since 1951 (AMS web site: Table 3).

Mel's research, over six decades, concentrated on the biology of *Crassostrea virginica* and its predator *Urosalpinx cinerea*. Mel believed that the biology and ecology of predator and prey were entwined and that one could not be understood without knowledge of the other. How does *U. cinerea* penetrate the shell of *C. virginica*? How do newly hatched *U. cinerea* find *C. virginica*? What are the structures and physiology of *U. cinerea* that allow it to bore a hole through the shell of *C. virginica* and other bivalves? Mel employed everything from simple field observations to x-ray microanalysis. His observations included sound recordings of the rasping of *U. cinerea* and cinematography (which can still be viewed on a web site). He was also among the first to apply scanning electron microscopy to the microstructure of the radula of *U. cinerea* and the shells of *C. virginica* and *Mytilus edulis* (Linnaeus, 1758). Mel identified the accessory boring organ (ABO) of the drills *U. cinerea* and *Eupleura caudate* (Say, 1822) and, through anatomical, histological, and histochemical methods, elucidated their structure and function in penetration of bivalve shells. Mel was able to link the shape of a bore hole with the snails that produced it even in paleontological specimens (Carriker and Yochelson 1968). Mel's studies also made use of histochemistry, and he examined the elemental analysis of major and minor trace

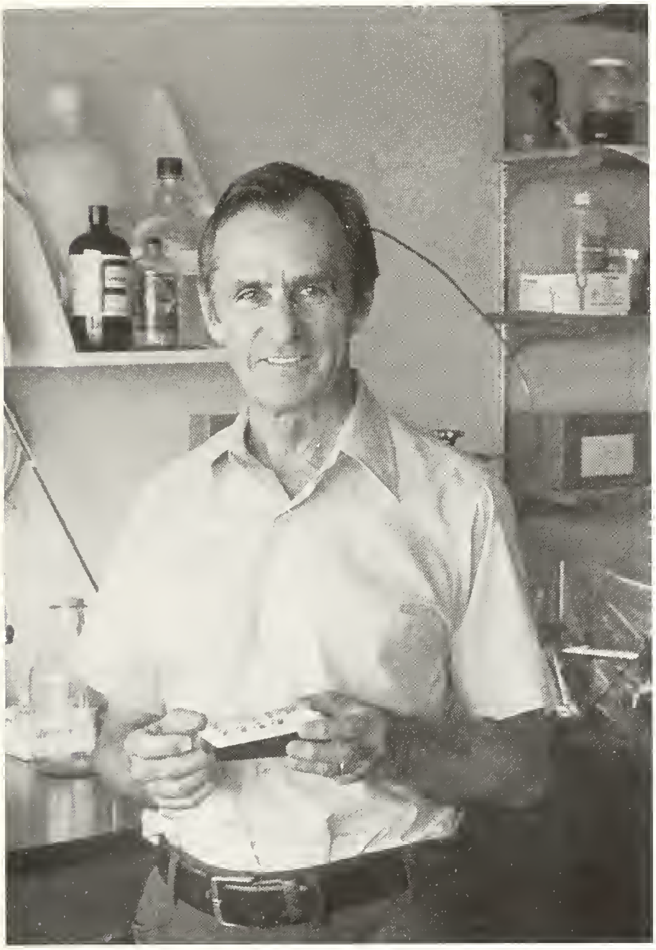

Figure 1. Mel Carriker in his laboratory early in his career at the University of Delaware, College of Marine Studies (now Graduate College of Marine and Earth Studies). University of Delaware stock photograph.

elements in oyster shell using a proton probe, developed by Charles P. Swann. Mel also studied chemoreception by *U. cinerea* and *E. caudata* with his student Betsy Brown and post-doctoral fellows Leslie G. Williams and Dan Rittschof. Mel continued to exercise his interest in estuarine pollution and its effects on the benthos, the invasion of coastal waters by exotic species and the impact of those invasions on commercially-valuable molluscan species. Mel summarized much of the results of his long study of oysters in Kennedy *et al.* (1996) and *Mercenaria mercenaria* in Kraeuther and Castagna (2001).

Mel served in many scientific organizations. Mel was particularly active in the National Shellfisheries Association (NSA), and in 1998 the Association founded a student research grant in his name. In the NSA, he served as Treasurer, Secretary, Vice-President, President, and Editor of the *Proceedings of the National Shellfisheries Association.* Mel was named an Honored Life Member of the Association in 1991. Mel was instrumental in the transformation of the *Proceedings of the National Shellfisheries Association* into the *Journal of Shellfish Research.* In 2005, Mel published a history of the association: *The Taming of the Oyster.* Mel was also active in the American Malacological Society in which he served as Vice-President, President, Member of Council, and was named an Honorary Life Member. Mel was instrumental in the transformation of the *Bulletin of the American Malacological Union* into the *American Malacological Bulletin* and served as a founding Associate Editor. At the most recent meeting of the AMS, a Carriker Student Research Grant program was also founded. He was a member of the Institute of Malacology, which publishes *Malacologia*, and he long served on the editorial board of the *Quarterly Review of Biology.* Other professional societies included the American Society of Zoologists, the New England Estuarine Research Federation (in which he was an Honorary Life Member), and the Atlantic Estuarine Research Federation.

Mel retired from the University of Delaware in 1985 at the age of 70 and was named Professor Emeritus. A symposium was held in his honor on the Lewes campus at which many of his friends and colleagues presented papers (Prezant and Counts 1985). Mel was so esteemed among his students that in 2001, to honor his 85th birthday, his students surprised Mel with "Carrikerfest", a celebration of his life to date [http://darc.cms.udel.edu/carrikerfest2001/cfestindex.html]. To further honor his contributions, his students and the university presented him with the Carriker Contemplative Garden just next to the shellfisheries laboratory. Mel, who walked to work on a daily basis, continued working at Lewes until two days before he suffered his stroke. During his emeritus years, Mel continued to submit annual activities reports to the Dean's office, although he was no longer required to do so. Dr. Nancy Targett, Dean of the College, noted that Mel had more productive years in retirement than some faculty members aspire to during their active career. During his retirement, Mel published 31 papers, 4 book chapters, and 4 books. Mel served as president of the Delaware Partners in the Americas in which he worked for closer scientific cooperation between the University and Panama. He also actively served the Association of Marine Laboratories of the Caribbean. By 2000, Mel had published over 160 professional papers (AMS web site: Table 4) and reports, 45 abstracts, and more than 255 presentations at scientific meetings, a significant portion during his "retirement" years. Mel continued to participate in professional meetings throughout his professional life (Fig. 2). As was true throughout his entire career, Mel deeply respected his students and colleagues. This has been recognized by the National Shellfisheries Association and the American Mala-

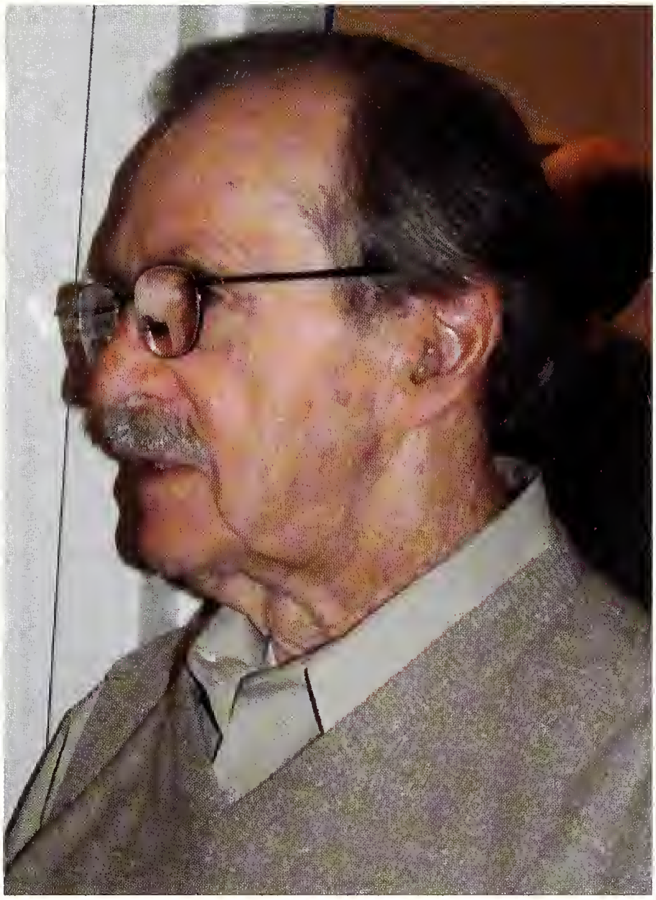

Figure 2. Mel Carriker at the March 2006 MidAtlantic Malacologists meeting at the Delaware Museum of Natural History. Photo by Robert Robertson.

cological Society and, joining those professional organizations in memorializing Mel's dedication to his students, the University of Delaware, College of Marine and Earth Studies has now established the Melbourne R. Carriker Student Fellowship Endowment.

Mel will be remembered for his many professional and scientific accomplishments but those of us who were honored to be his friends and students will always treasure the warmth of his friendship, encouragement, high standard of professional conduct, and devotion to the advancement of science. All of us who knew Mel Carriker are better for it.

ACKNOWLEDGEMENTS

We thank Dr. Robert Robertson (ANSP, retired) for photographs of Mel and Dr. Nancy Targett, Dean, Graduate College of Marine and Earth Sciences, University of Delaware, Peggy Conlon, and staff for assistance in assembling Mel's records and bibliography.

Note: A full listing of Mel's graduate students (including thesis and dissertation topics), a listing of those graduate students who had Mel sit on their research committees, and his complete bibliography can be found at the web site of the American Malacological Society: http://www.malacological.org/

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AMERICAN MALACOLOGICAL SOCIETY, INC
FINANCIAL REPORT
General Accounts
2005 Income and Expenses

TOTAL ASSETS (January 1, 2005)			$170,765.82
INCOME			$34,426.78
Membership Dues		14,025.00	
Membership Dues (2002)	20.00		
Membership Dues (2003)	489.00		
Membership Dues (2004)	1,622.00		
Membership Dues (2005)	8,806.00		
Membership Dues (2006)	2,031.00		
Membership Dues (2007)	1,009.00		
Membership Dues (2008)	48.00		
Interest and Dividends from Endowment		3,632.21	
Capital Gains Distribution	10.98		
Life Membership Fund	275.74		
Symposium & Student Fund	3,345.49		
Publications Income		9,481.36	
AMB Subscriptions	2,129.00		
AMB Page Charges	4,055.00		
AMB Back Issues	608.00		
AMB Reprint Charges	1,641.00		
AMB Postage & Misc. Income	1,048.36		
Donations		1,516.00	
Symposium Endowment Fund	255.00		
Student Endowment Fund	1,161.00		
Shell Museum Hurricane Fund	100.00		
Income from Annual Meeting		5,772.21	
EXPENSES			$36,856.79
Treasurer and Secretary Office Expenses		172.33	
Affiliate Memberships		225.00	
Banking & Credit Card Fees		681.77	
Incorporation & Registration Fees		95.00	
Insurance/Bond Fees		552.00	
Website Expenses		886.76	
Annual Meeting Deposit & Symposium Expenses		7,400.00	
Publication Expenses		12,225.83	
AMB (20 (1/2))	10,901.68		
Reprints	372.82		
Managing Editor Travel	859.78		
Postage	63.55		
Overpayment refunds	28.00		
Student Research Grants		3,000.00	
Travel Expenses for Officers		3,618.10	
Student Paper Awards		500.00	
Hurricane Relief		7,500.00	
NET LOSS in 2005			$2,430.01
TOTAL ASSETS (December 31, 2005)			$170,097.82

**Includes capital gains and losses in endowment portfolios which fluctuate with the market.

AMERICAN MALACOLOGICAL SOCIETY, INC
FINANCIAL REPORT
General Accounts
2006 Income and Expenses

TOTAL ASSETS (January 1, 2006)			170,097.82
INCOME			$58,370.04
Membership Dues		13,783.00	
Membership Dues (2004)	80.00		
Membership Dues (2005)	737.00		
Membership Dues (2006)	8,608.00		
Membership Dues (2007)	2,575.00		
Membership Dues (2008)	1,687.00		
Membership Dues (2009)	48.00		
Membership Dues (2010)	48.00		
Interest and Dividends from Endowment		4,087.54	
Life Membership Fund	310.49		
Symposium & Student Fund	3,777.05		
Publications Income		9,682.89	
AMB Subscriptions	5,176.27		
AMB Page Charges	1,896.00		
AMB Back Issues	96.00		
AMB Reprint Charges	533.00		
AMB and Book Royalties	1,511.62		
AMB Postage & Misc. Income	470.00		
Donations		3,769.00	
Symposium Endowment Fund	20.00		
Student Endowment Fund	300.00		
Student Fund from Auction	3,449.00		
Income from Annual Meeting		27,047.61	
Field trip income	395.00		
Registration and other income	26,652.61		
EXPENSES			$40,499.50
Treasurer and Secretary Office Expenses		339.51	
Affiliate Memberships		325.00	
Banking & Credit Card Fees		840.19	
Incorporation & Registration Fees		45.00	
Insurance/Bond Fees		1,037.00	
Website Expenses		110.35	
Annual Meeting & Symposium Expenses		23,450.64	
Publication Expenses		12,369.12	
AMB (21(1/2))	8,817.63		
Reprints	658.97		
Sturm Book	1,365.08		
Managing Editor Travel		1,474.93	
Misc. postage etc		52.51	
Student Research Grants		2,000.00	
Travel Expenses for Officers		3,363.66	
Student Paper Awards		500.00	
Net Gain in 2006			$13,989.57
TOTAL ASSETS (December 31, 2006)			$193,587.74

**Includes capital gains and losses in endowment portfolios which fluctuate with the market.

INDEX TO VOLUME 23

AUTHOR INDEX

Baur, B. 23: 157
Chase, R. 23: 183
Coan, E. V. 23: 1
Counts, C. L., III 23: 191
Davison, A. 23: 173
Fortunato, H. 23: 33
Gallardo, C. S. 23: 17
Herbert, G. S. 23: 17
De Visser, J. A. G. M. 23: 113
Jansen, R. F. 23: 113
Kabat, A. R. 23: 1
Koene, J. M. 23: 113
Krug, P. J. 23: 99
Leonard, J. L. 23: 79, 121
Merle, D. 23: 17
Montagne-Wajer, K. 23: 113
Mordan, P. 23: 173
Ohtaki, H. 23: 81
Pearse, J. S. 23: 121
Prezant, R. S. 23: 191
Reise, H. 23: 137
Robertson, R. 23: 11
Simone, L. R. L. 23: 43
Takeuchi, M. 23: 81
Ter Maat, A. 23: 113
Tomiyama, K. 23: 81
Ward, J. E. 23: 191
Westfall, J. A. 23: 121
Yusa, Y. 23: 89
Zonneveld, C. 23: 113

PRIMARY MOLLUSCAN TAXA INDEX

[first occurrence in each paper recorded, new taxa in bold]

Abyssochrysidae 23: 11
Abyssochrysos 23: 11
Acanthina 23: 20, 72
Acanthotrophon 23: 19
Acavidae 23: 176
Achatina 23: 176
Achatinidae 23: 176
adolphi, Deroceras 23: 140
adspersa, Tenellia 23: 100
Aeolidiella 23: 13
Aeolidiidae 23: 13
Aeolidioidea 23: 13
Agathylla 23: 176
agreste, Deroceras 23: 138
agrestis, Agriolimax 23: 139
Agriolimacidae 23: 137, 176
Agriolimax 23: 138
Albinaria 23: 176
Alderia 23: 99
Allogona 23: 175
Alopiinae 23: 177
alveata, Caribiella 23: 20
amanoi, Satsuma 23: 177
Amphidromus 23: 174
Amphissa 23: 35
Ampullariidae 23: 93
Ancilla 23: 72
ancilla, Fulmentum 23: 43
ancilla, Pseudoliva 23: 54
ancilla, Sylvanocochlis 23: 54
Anguispira 23: 177
antillarum, Nassarius 23: 34
antipodarum, Potamopyrgus 23: 91
Aplysia 23: 79
arbustorum, Arianta 23: 80, 138, 157, 174
Archachatina 23: 176
Architectonicidae 23: 12
arenaria, Mya 23: 96
argentea, Cotonopsis 23: 34
Arianta 23: 175
Ariolimax 23: 121, 176
Arion 23: 176
Arionidae 23: 121, 151, 176
Ariophanta 23: 176
Ariophantidae 23: 176
Ashmunella 23: 175
aspersa, Helix 23: 118, 160, 174, 183
aspersum, Cornu 23: 150
aspersus, Cantareus 23: 174, 183
atafona, Benthobia 23: 43
ater, Viviparus 23: 91
Atlanta 23: 12
Atlantidae 23: 12
australis, Eburna 23: 45
australis, Zemira 23: 43
avellana, Cronia 23: 21
avenacea, Chrondrina 23: 129
Balea 23: 176
Baleinae 23: 176
Basommatophora 23: 113
Bembicium 23: 11
Benthobia 23: 43
Bertia 23: 176
bistimulatum, Deroceras 23: 141
Bittiolum 23: 12
Bittium 23: 12
Bivetiella 23: 72
Bizetiella 23: 19
Boonea 23: 12
brachyphallus, Ariolimax 23: 122
Bradybaena 23: 175
Bradybaenidae 23: 175
brevispina, Murex 23: 19
buccineus, Xymenopsis 23: 19
Buccinidae 23: 35, 43
Buccinoidea 23: 33
Buccinorbis 23: 43
Buccinum 23: 54
bureschi, Deroceras 23: 138
buttoni, Aphallarion 23: 122
buttoni, Ariolimax 23: 121
Caenogastropoda 23: 43
californica, Aplysia 23: 107
californicus, Ariolimax 23: 121, 138
callidegenita, Haminaea 23: 100
calliglypta, Anachis 23: 35

calliglypta, Cosmioconcha 23: 34
Calyptraeoidea 23: 43
Camaenidae 23: 175
Campaniloidea 23: 12
canaliculata, Pomacea 23: 89
Cancellariidae 23: 43
Cantareus 23: 175
Caracolus 23: 175
carica, Busycon 23: 91
Carinariidae 23: 12
cassidiformis, Xanthochorus 23: 21
castanea, Angustassiminea 23: 90
Catinella 23: 177
caucasicum, Deroceras 23: 138
caudate, Eupleura 23: 192
cauze, Elysia 23: 100
Cavoliniidae 23: 12
Cavolinioidea 23: 12
Cenchritis 23: 11
Cepaea 23: 175
Cepolis 23: 175
Cerastidae 23: 176
Ceriidae 23: 176
Cerion 23: 176
Cerithiidae 23: 12
Cerithioidea 23: 12, 43
Cerithiopsidae 23: 11
Cerithiopsis 23: 11
Cerithiopsoidea 23: 11
Cerithium 23: 12
Chicocenebra 23: 20
Chicopinnatus 23: 30
Chicoreus 23: 30
chlorotica, Elysia 23: 100
Chondrinidae 23: 176
Chorus 23: 20
christae, Deroceras 23: 140
Chytra 23: 12
cincinnatiensis, Pomatiopsis 23: 92
cinerea, Urosalpinx 23: 17, 192
cingulata, Cerithideopsilla 23: 81
Cinnalepeta 23: 12
Clathrus 23: 11
Clausilia 23: 176
Clausiliidae 23: 176
Clausiliinae 23: 177
Clionoidea 23: 12
Clithon 23: 12
Cochlicella 23: 175
Cochlodina 23: 176
Columbellidae 23: 33
columbianus, Ariolimax 23: 121
complexirhyna, Benthobia 23: 43
concentricus, Latirus 23: 34
concholepas, Concholepas 23: 17
Conoidea 23: 43
convexa, Crepidula 23: 92
coralium, Clypeomorus 23: 81
Corbicula 23: 91
cornuarietis, Marisa 23: 90
Cosmioconcha 23: 33
Cotonopsis 23: 33
crassa, Pseudoliva 23: 76
crassiparva, Cotonopsis 23: 34
Crassispirinae 23: 2
Crassostrea 23: 89
crassulnata, Cronia 23: 18
Crepidula 23: 92
Crucibranchaea 23: 12
Cryptomastix 23: 175
cumingi, Batillaria 23: 81
Cypraea 23: 11
Cypraeidae 23: 11
Cypraeoidea 23: 11, 43
Delima 23: 176
Dendropoma 23: 12
Deroceras 23: 137, 176
deroyae, Cotonopsis 23: 34
Diacria 23: 12
Diala 23: 12
Dialidae 23: 12
Discidae 23: 177
djadjariensis, Cerithideopsilla 23: 81
dolichophallus, Ariolimax 23: 121, 177
dorri, Canidia 23: 68
dorri, Nassodonta 23: 43
Echininus 23: 11
edentula, Cotonopsis 23: 34
edulis, Mytilus 23: 192, 195
elata, Drupella 23: 18
Elimia 23: 12, 92
Elisolimax 23: 176
Enidae 23: 177
Eobania 23: 175
Epitoniidae 23: 11
Epitonioidea 23: 11
Epitonium 23: 11
Ergalataxinae 23: 20
Erronea 23: 11
esmeraldensis, Cotonopsis 23: 34
Euglandina 23: 177
Euhadra 23: 174
Eulimidae 23: 12
Euphaedusa 23: 176
faba, Clithon 23: 81
Fargoa 23: 12
fasciatus, Liguus 23: 174
Fasciolariidae 23: 35, 72
fatrense, Deroceras 23: 138
Faunus 23: 12
Favartia 23: 18
festiva, Reticunassa 23: 81
Finella 23: 12
floridensis, Orthalicus 23: 174
fornicata, Crepidula 23: 92
fulica, Lissachatina 23: 174
Fulmentum 23: 54
Furcopenis 23: 139
Fusitriton 23: 11
galea, Tonna 23: 76
galloprovincialis, Mytilus 23: 95
Gastrodontidae 23: 176
Gastropoda 23: 81
Geomalacus 23: 176
geversianus, Trophon 23: 17
gigas, Crassostrea 23: 91
giustianum, Deroceras 23: 140
glandulosum, Deroceras 23: 140
gorgonium, Deroceras 23: 138, 140
grandis, Typhisala 23: 19
gubbi, Chicoreus 23: 20
Gymnarion 23: 176
Halolimnohelix 23: 175
Haplotrema 23: 177
Haplotrematidae 23: 177
harpa, Neothais 23: 18
haustorium, Haustrum 23: 19
Haustrinae 23: 18
Hedylopsoidea 23: 13
Helicidae 23: 157, 174
helicoidale, Deroceras 23: 140
Helicoidea 23: 174
Helicophanta 23: 176
Helix 23: 79, 175
Helminthoglypta 23: 175
Helminthoglyptidae 23: 175
Hemiplecta 23: 176
Herilla 23: 176
heynemanni, Trichotoxon 23: 174
hirundo, Cotonopsis 23: 34

Humboldtiana 23: 175
Hygromiidae 23: 175
Hypsogastropoda 23: 73
Hyriidae 23: 95
Ianthina 23: 11
Iberus 23: 175
ikaria, Deroceras 23: 140
indentata, Aspella 23: 19
insignis, Nassa 23: 68
Iolaea 23: 12
jaliscana, Cotonopsis 23: 34
Janthina 23: 11
Janthinidae 23: 11
Janthinoidea 23: 11
japonica, Assiminea 23: 90
Laciniaria 23: 176
laeve, Deroceras 23: 138
laevigata, Strombina 23: 35
laevis, Agriolimax 23: 139
laqueatus, Chicopinnatus 23: 19
lateralis, Mulinia 23: 96
Lavigeria 23: 12
Leucozonia 23: 72
Liguus 23: 177
Limacidae 23: 176
Limacina 23: 12
Limacinidae 23: 12
Limacoidea 23: 174
Limacus 23: 176
Limapontiidae 23: 101
Limax 23: 153, 176
Limnotrochus 23: 12
lindae, Cotonopsis 23: 34
lineata, Pyrula 23: 61
lineatum, Melapium 23: 43
Liolytopelte 23: 137
Litiopidae 23: 13
Littoraria 23: 11
Littorina 23: 11
Littorinidae 23: 11
Littorinoidea 23: 11
lombricoides, Deroceras 23: 138
Lophiotominae 23: 2
Loxonematoidea 23: 11
Luchuphaedusa 23: 176
luteostoma, Nassarius 23: 34
Lymnaeidae 23: 113
Macrochlamys 23: 176
Macrogastra 23: 176
Macron 23: 72
maculosa, Tonna 23: 76
Mandarina 23: 175
margaritifera, Margaritifera 23: 92
Margartiferidae 23: 95
marmoratus, Iberus 23: 174
Mastus 23: 177
maximus, Limax 23: 79, 173
mazatlantica, Pinctada 23: 91
Medora 23: 176
Melanopsidae 23: 12
Melanopsinae 23: 12
Melapiidae 23: 68
Melapium 23: 43
Melarhaphe 23: 11
mendozana, Cotonopsis 23: 34
mercenaria, Mercenaria 23: 192
Mesodon 23: 175
Mesomphix 23: 176
Microparmarion 23: 176
Milacidae 23: 176
minoicum, Deroceras 23: 140
modesta, Alderia 23: 99
modesta, Cosmioconcha 23: 34
modestum, Buccinum 23: 35
Monacha 23: 175
Monadenia 23: 175
monfilsi, Cotonopsis 23: 34
Mudalia 23: 12
multiformis, Batillaria 23: 81
Murexiella 23: 18
Murexsul 23: 18
Muricidae 23: 17, 43
Muricinae 23: 17
Muricoidea 23: 43
Muricopsinae 23: 17
Muricopsis 23: 18
Mysorelloides 23: 12
Mytildae 23: 95
Mytilus 23: 89
Nassariidae 23: 35, 43
Nassodonta 23: 68
Naticoidea 23: 75
nemoralis, Cepaea 23: 159, 174
Neogastropoda 23: 33, 74
Neohelix 23: 175
Neostyriaca 23: 176
Neripteron 23: 12
Nerita 23: 12
Neritidae 23: 12
Neritilia 23: 12
Neritiliidae 23: 12
Neritina 23: 12
Neritoidea 23: 12
nitens, Cosmioconcha 23: 34
nitens, Fusus 23: 35
Nitidiscala 23: 11
nitidum, Deroceras 23: 138
Nodilittorina 23: 11
Nucella 23: 19
ocellifera, Costasiella 23: 100
Ocenebra 23: 19
Ocenebrinae 23: 17
Ocinebrina 23: 19
octagonus, Murexsul 23: 19
oculifera, Aplysia 23: 107
Odostomella 23: 12
oertzeni, Deroceras 23: 140
Olividae 23: 43
Opalia 23: 11
Opisthobranchia 23: 99
Oreohelicidae 23: 177
Oreohelix 23: 174
Orthalicidae 23: 174
ostrina, Nucella 23: 21
oualaniensis, Clithon 23: 81
Oxychilus 23: 176
Oxyloma 23: 177
palmeri, Cosmioconcha 23: 34
Paludomidae 23: 12
Palustorina 23: 12
panacostaricensis, Cotonopsis 23: 34
panormitanum, Deroceras 23: 137
Paramelania 23: 12
Parhedylidae 23: 13
Parthenina 23: 12
Partula 23: 177
Partulidae 23: 177
parvula, Cosmioconcha 23: 34
Paryphanta 23: 177
pergracilis, Cosmioconcha 23: 34
perpicta, Columbella 23: 35
perpicta, Mitrella 23: 35
perrugata, Urosalpinx 23: 20
Phenacolepadidae 23: 12
Philomycidae 23: 174
Philomycus 23: 177
phuketensis, Cotonopsis 23: 34
Pisulina 23: 12
planarioides, Deroceras 23: 138
Plesiotrochidae 23: 12

Plesiotrochus 23: 12
Pleuroceridae 23: 12
Pleurodonte 23: 175
Pneumodermatidae 23: 12
Polydontes 23: 175
Polygyra 23: 175
Polygyridae 23: 175
Polymita 23: 175
pomatia, Helix 23: 159, 184
pomum, Chicoreus 23: 17
pomum, Phyllonotus 23: 17
Pontohedyle 23: 13
Potamididae 23: 12, 81
praecox, Deroceras 23: 138, 140
Pseudoliva 23: 43
Pseudolividae 23: 43
Pseudolivinae 23: 43
Pseudomelatominae 23: 2
Ptenoglossa 23: 12
Pterosoma 23: 12
Pterotracheoidea 23: 12
Pulmonata 23: 137
pupa, Mastus 23: 177
Puperita 23: 12
pusilla, Vertigo 23: 133
Pygmaepterys 23: 18
Pyramidella 23: 12
Pyramidellidae 23: 12
Pyramidelloidea 23: 12
quadrata, Sinotaia 23: 91
radwini, Cotonopsis 23: 34
Ranellidae 23: 11
Rapaninae 23: 17
rehderi, Cosmioconcha 23: 34
rethimnonensis, Deroceras 23: 138
reticulatum, Deroceras 23: 138
Reymondia 23: 12
rhizophorarum, Cerithidea 23: 81
Rhytididae 23: 177
ringens, Cantharus 23: 34
rodnae, Deroceras 23: 138
Ruthenica 23: 176
Sacoglossa 23: 99
salebrosa, Vitularia 23: 17
sandai, Euhadra 23: 177
Satsuma 23: 175
Scala 23: 11
Scaliolidae 23: 12
Seila 23: 11
semicarinata, Goniobasis 23: 92
Semilimax 23: 176
Semisulcospira 23: 12
senegalensis, Siratus 23: 43
sepimentum, Buccinum 23: 54
Septaria 23: 12
Serpulorbis 23: 12
Sheldonia 23: 176
Siratus 23: 72
skoglundae, Cotonopsis 23: 34
Solatopupa 23: 176
Sonorella 23: 175
sorenseni, Acanthotrophon 23: 20
Spekia 23: 12
Spiraxidae 23: 177
stagnalis, Lymnaea 23: 79, 113, 153, 191
Stanleya 23: 12
Stenotrema 23: 176
Stereophaedusa 23: 176
Stormsia 23: 12
stramineus, Ariolimax 23: 132
Strepturidae 23:
Strombina 23: 33
Stromboidea 23: 44
Strophocheilidae 23: 177
Strophocheilus 23: 177
sturanyi, Deroceras 23: 138
Stylommatophora 23: 11, 121, 183
subagreste, Deroceras 23: 141
Succinea 23: 173
Succineidae 23: 177
suteri, Cotonopsis 23: 34
Sylvanocochlis 23: 43
Tacheocampylaea 23: 175
Tandonia 23: 176
Tanganyicia 23: 12
Tectarius 23: 11
Terebralia 23: 12
Thais 23: 19
Theba 23: 175
Theliostyla 23: 12
Thiara 23: 12
Thiaridae 23: 12
timida, Elysia 23: 100
Tiphobia 23: 12
Tonnoidea 23: 11, 43
torrefactus, Chicoreus 23: 22
Trichotoxon 23: 176
Trilobopsis 23: 176
Triodopsis 23: 176
Triphora 23: 11
Triphoridae 23: 11
Triphoroidea 23: 11
Triplex 23: 30
Tripterotyphinae 23: 18
tritoniformis, Agnewia 23: 21
Trochomorphidae 23: 176
Trophon 23: 30
Trophoninae 23: 17
trossulus, Mytilus 23: 95
truncatus, Bulinus 23: 79, 133
turcicum, Deroceras 23: 138
Turrina 23: 33
turrita, Cotonopsis 23: 34
Turritella 23: 12
Turritellidae 23: 12
Typhinae 23: 18
Unela 23: 13
Unionidae 23: 95
Urocyclidae 23: 176
Vallonia 23: 177
Valloniidae 23: 177
Veneridae 23: 95
Ventridens 23: 176
Vermetidae 23: 12
Vermetoidea 23: 12
Vermetus 23: 12
verrucicornis, Berghia 23: 100
Vertiginidae 23: 177
Vertigo 23: 177
Vespericola 23: 176
Vinundu 23: 12
virginica, Crassostrea 23: 94, 192
Viriola 23: 11
Vitrinidae 23: 176
Vitrinobrachium 23: 176
Vittina 23: 12
Vitularia 23: 19
Viviparus 23: 92
willowi, Alderia 23: 99
Xanthochorus 23: 20
Zachrysia 23: 175
zebrina, Pseudoliva 23: 76
Zebrinops 23: 176
Zemira 23: 43
Zonitidae 23: 176
Zonitoides 23: 130

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Poecilogony and larval ecology in the gastropod genus *Alderia*. **PATRICK J. KRUG** 99

Food intake, growth, and reproduction as affected by day length and food availability in the pond snail *Lymnaea stagnalis*. **ANDRIES TER MAAT, COR ZONNEVELD, J. ARJAN G.M. DE VISSER, RENÉ F. JANSEN, KORA MONTAGNE-WAJER, and JORIS M. KOENE** 113

Phally polymorphism and reproductive biology in *Ariolimax (Ariolimax) buttoni* (Pilsbry and Vanatta, 1896) (Stylommatophora: Arionidae). **JANET L. LEONARD, JANE A. WESTFALL, and JOHN S. PEARSE** 121

A review of mating behavior in slugs of the genus *Deroceras* (Pulmonata: Agriolimacidae). **HEIKE REISE** 137

Reproductive biology and mating conflict in the simultaneously hermaphroditic land snail *Arianta arbustorum*. **BRUNO BAUR** 157

A literature database on the mating behavior of stylommatophoran land snails and slugs. **ANGUS DAVISON and PETER MORDAN** 173

The function of dart shooting in helicid snails. **RONALD CHASE** 183

Meeting Announcement 190

Melbourne Romaine Carriker: 25 February 1915 – 25 February 2007 An Appreciation. **CLEMENT L. COUNTS, III, ROBERT S. PREZANT, and J. EVAN WARD** 191

Financial Report 195

Index to Vol. 23 197

Membership Form 201

Information for Contributors 203

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401
A513
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AMERICAN MALACOLOGICAL BULLETIN

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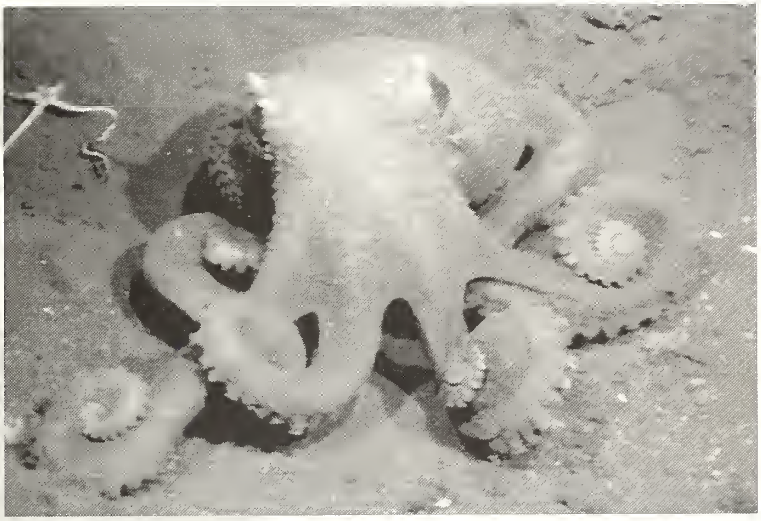
VOLUME 24 21 MARCH 2008 NUMBER 1/2



Introduction to the symposium "Cephalopods: A behavioral perspective."
JENNIFER A. MATHER 1

Chambered nautilus (*Nautilus pompilius pompilius*) responds to underwater vibrations.
CHRISTIAN P. SOUCIER and JENNIFER A. BASIL 3

Octopus sucker-arm coordination in grasping and manipulation. **FRANK W. GRASSO** 13

Short-term pain for long-term gain: A hypothetical role for the mantle in coleoid cephalopod circulation. **ALISON J. KING and SHELLEY A. ADAMO** 25

A new approach to octopuses' body pattern analysis: A framework for taxonomy and behavioral studies. **TATIANA. S. LEITE and JENNIFER A. MATHER** 31

Observations of deep-sea octopodid behavior from undersea vehicles. **JANET R. VOIGHT** 43

To boldly go where no mollusc has gone before: Personality, play, thinking, and consciousness in cephalopods. **JENNIFER A. MATHER** 51

Freshwater snails (Mollusca: Gastropoda) from the Commonwealth of Dominica with a discussion of their roles in the transmission of parasites.
WILL K. REEVES, ROBERT T. DILLON, JR., and GREGORY A. DASCH 59

continued on back cover

Cover photo: *Graneledone* Joubin, 1918 from Baby Bare, Cascadia Basin, North Pacific Ocean from Voight

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Seattle, Washington 98195
USA

Dianna Padilla
Department of Ecology and Evolution
Stony Brook University
Stony Brook, New York 11749-5245
USA

Roland C. Anderson
The Seattle Aquarium
1483 Alaskan Way
Seattle, Washington 98101
USA

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Louisiana State University
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AMERICAN MALACOLOGICAL BULLETIN

CONTENTS

VOLUME 24 | NUMBER 1/2

APR 24
LIBRARIES

Introduction to the symposium "Cephalopods: A behavioral perspective." **JENNIFER A. MATHER** 1

Chambered nautilus (*Nautilus pompilius pompilius*) responds to underwater vibrations. **CHRISTIAN P. SOUCIER and JENNIFER A. BASIL** 3

Octopus sucker-arm coordination in grasping and manipulation. **FRANK W. GRASSO** 13

Short-term pain for long-term gain: A hypothetical role for the mantle in coleoid cephalopod circulation. **ALISON J. KING and SHELLEY A. ADAMO** 25

A new approach to octopuses' body pattern analysis: A framework for taxonomy and behavioral studies. **TATIANA. S. LEITE and JENNIFER A. MATHER** 31

Observations of deep-sea octopodid behavior from undersea vehicles. **JANET R. VOIGHT** 43

To boldly go where no mollusc has gone before: Personality, play, thinking, and consciousness in cephalopods. **JENNIFER A. MATHER** 51

Freshwater snails (Mollusca: Gastropoda) from the Commonwealth of Dominica with a discussion of their roles in the transmission of parasites. **WILL K. REEVES, ROBERT T. DILLON, JR., and GREGORY A. DASCH** 59

Patterns of activity cycles in juvenile California two-spot octopuses (*Octopus bimaculoides*). **DAVID L. SINN** 65

Discovery of the South African polyplacophoran *Stenosemus simplicissimus* (Thiele, 1906) (Mollusca, Polyplacophora, Ischnochitonidae) in the Southern Ocean. **ENRICO SCHWABE** 71

Imposex level and penis malformation in *Hexaplex trunculus* from the Tunisian coast. **YOUSSEF LAHBIB, SAMI ABIDLI, and NAJOUA TRIGUI EL MENIF** 79

Threatened Bliss Rapids snail's susceptibility to desiccation: Potential impact from hydroelectric facilities. **DAVID C. RICHARDS and TRISTAN D. ARRINGTON** 91

Field observations of the nocturnal mantle-flap lure of *Lampsilis teres*. **ANDREW LEE RYPEL** 97

Meta-analysis of the relationship between salinity and molluscs in tidal river estuaries of southwest Florida, U.S.A. **PAUL A. MONTAGNA, ERNEST D. ESTEVEZ, TERRY A. PALMER, and MICHAEL S. FLANNERY** 101

Research Note: Giant African snail, *Achatina fulica*, as a snail predator. **WALLACE M. MEYER III, KENNETH A. HAYES, and AMANDA L. MEYER** 117

Research Note: Life history and host fish identification for *Fusconaia burkei* and *Pleurobema strodeanum* (Bivalvia: Unionidae). **MEGAN P. WHITE, HOLLY N. BLALOCK-HEROD, and PAUL M. STEWART** 121

Index to Vol. 24 127

Membership Form 130

Information for Contributors 132

Meeting Announcement 134

Amer. Malac. Bull. **24**: 1

Introduction to the symposium "Cephalopods: A behavioral perspective"*

Jennifer A. Mather

Department of Psychology, University of Lethbridge, 4401 University Drive, Lethbridge, Alberta T1K 3M4, Canada, mather@uleth.ca

Behavior is not an area we usually associate with molluscs, and one tends to think instead of vertebrates, especially mammals. Yet when we do think of molluscan behavior, it is the cephalopods that come to mind. With their large centralized brain, reputed high intelligence, efficient physiology, and complex motor output, cephalopods have an excellent basis for complex behavior. Despite this capacity, cephalopod behavior is little known and not well explored, and the authors in this symposium, especially the paper collection, attempt to shine light into various corners with a wide variety of cephalopod subjects.

One of the simplest aspects of behavior is sensory reception, and one of the 'simplest' systems and most molluscan-general is that found in *Nautilus* Linnaeus, 1758. Soucier and Basil discuss a pioneering laboratory investigation of tactile sensitivity in the nautiloids; clearly these deep-sea animals should rely on non-visual information much of the time, but only their chemical sensing has been well investigated. Now that their mechanical reception has been established, further research will no doubt look more at its use in natural situations and the limits of and receptors for its sensitivity.

The programming of motor output, the root of behavior, is similarly simple on the surface. Grasso has tackled the motor output of suckers of *Octopus* Cuvier, 1797 and their combinations to produce actions on the environment. While movement ought to be simple, the use and coordination of hundreds of suckers turns out to be, as befits the complexity of neural support of the suckers, both complex and variable. How much of this programming is central and how much peripheral as well as how the 'reflex' arm control system can perform such complex maneuvers remains to be investigated; again the foundation has been laid for further investigation.

Behavior is linked to the underlying physiology of the animal, and the thoughtful paper by King and Adamo makes sense of the paradoxes in the combination of *Sepia* Linnaeus, 1758 cuttlefish linkage of mantle contraction and blood circulation. The motor action of mantle contraction has a major effect on the circulation of blood through this area, and the authors evaluate why the particular patterns of blood flow during this major event occur. More of such behavior-physiological linkage is needed, and the King and Adamo paper is a welcome start.

One unique, coleoid cephalopod motor system is responsible for the chromatophore system that produces skin patterns and colors, but its complexity means that it is often characterized only informally. Leite and Mather use a computerized data analysis approach to build the repertoire of one *Octopus* species. Such characterization offers insight into the neural production of patterns and pattern complexity on the skin; in addition, this approach may assist us in taxonomic investigation of the species complex of *Octopus vulgaris* Cuvier, 1797.

Behavior gives us insights into physiology and ecology of animals, and behavior of deep-sea octopods in underwater videos is the subject of the paper by Voigt. Because humans are very limited in their activities in the deep sea, cephalopod research has focused on the easily available near-shore and near-surface species of *Octopus*, *Sepia*, and *Loligo* Lamarck, 1798. Thus, Voight's insight into how these deep-sea and little-known animals behave is particularly welcome.

The most complex areas of behavior are the emergent aspects such as play, personality, and cognition, studied mainly in *Octopus* so far. Mather covers the research in these areas and suggests that we have much to learn about the intelligence, cognitive capacity, and even possible consciousness in cephalopods. She challenges us to look at behavior of molluscs, particularly in but not limited to cephalopods, for greater underlying subtly and complexity than we have assumed so far.

In addition to these published papers, other symposium participants presented work on a range of interesting aspects of cephalopod behavior. Huffard discussed octopus mating strategies for *Abdopus* Norman and Finn, 2001; Cosgrove discussed the brooding behavior of *Enteroctopus* Rochebrune and Mabille, 1889. Again with *Enteroctopus dofleini* (Wülker, 1910) as a model, Lyons and Scheel discussed the ecological impact and movement of octopuses in their natural environment. Finally, Williams looked at the chemical defenses of hatchling *Hapalochlaena* Robson, 1929, and Bush discussed why deep-sea squid might ink into the dark.

I would like to thank Roland Anderson of the Seattle Aquarium, for requesting the symposium and assisting in its assembly, and the helpful reviewers and patient authors who worked through all the revisions.

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* From the symposium "Cephalopods: A behavioral perspective" presented at the joint meeting of the American Malacological Society and Western Society of Malacologists, held 29 July to 3 August 2006 in Seattle, Washington.

Amer. Malac. Bull. **24**: 3-11

Chambered nautilus (*Nautilus pompilius pompilius*) responds to underwater vibrations*

Christian P. Soucier[1] and Jennifer A. Basil

Evolution, Ecology, and Behavior Program, City University of New York–Graduate Center, Department of Biology, CUNY Brooklyn College, 2900 Bedford Avenue, Brooklyn, New York 11210, U.S.A., CSoucier@brooklyn.cuny.edu and JBasil@brooklyn.cuny.edu

Abstract: The deep-water cephalopod *Nautilus pompilius pompilius* Linnaeus, 1758 may benefit from detecting potential signals such as mechanical and acoustical stimuli in its dark habitat where visual information is often limited. Here we examined whether specimens of chambered nautilus are capable of responding to waterborne vibration—a sensory mechanism that has yet to be investigated. We measured the ventilation rate of animals responding to a vibrating bead that produced a range of displacements and velocities. We found that nautiluses do indeed respond to underwater acoustical stimuli, decreasing their ventilation in the presence of a vibratory stimulus. Vibrations resulting from large-bead displacements and high source-velocities caused the animals to decrease their ventilation the most. Stimuli <20 cm from the animals caused a further reduction in their ventilation rates than those at greater distances. These nocturnal animals, living in dark conditions where visual information is often limited, may benefit from including vibrations in the suite of stimuli to which they can respond.

Key words: cephalopods, acoustics, behavior, ventilation, source-displacement

Organisms must cope with a variety of stimuli in the marine environment, and the ability to process this information may contribute to both survival and reproduction. Because the marine environment is dominated by mechanical and acoustical energies, such as water currents or vibrations that may eventually be converted to sound waves, it is a reasonable assumption that many organisms, including *Nautilus pompilius pompilius* Linnaeus, 1758, may benefit from the ability to detect and respond to these varying types of stimuli.

In the last three decades, researchers have identified the variety of sensory systems that contribute to the survival and functional ecology of the chambered nautilus (*e.g.*, Budelmann and Tu 1997). *Nautilus pompilius pompilius* has served as a model in studies of olfaction, vision, and equilibrium reception. Nautiluses, although predominantly chemotactic, are capable of using many sensory systems to complete basic survival tasks (*vision*: Muntz 1991, 1994a, 1994b, *equilibrium reception:* Budelmann 1977, Neumeister and Budelmann 1997, *olfaction*: Basil *et al.* 2000, 2002, 2005). Here we demonstrate that *Nautilus pompilius* is also capable of detecting and responding to underwater vibrational stimuli.

Nautilus pompilius is considered to be one of the oldest members of the class Cephalopoda (phylum Mollusca). Presently, the genus represents less than 1% of the entire cephalopod assemblage (Wood and O'Dor 2000). Nautiluses are the only extant hard-shelled cephalopod, and are therefore commonly used as a modern analog of the ellesmeroceratids, an ancestral lineage that dates back *ca.* 500 Ma (Ward 1987, Wray *et al.* 1995, Ward and Saunders 1997). Nautiluses are bottom dwellers but are not completely restricted to the sediment (nektobenthic). They make daily vertical migrations at dawn and dusk along coral reef slopes throughout the Indo-Pacific, including the Philippines, Palau, Fiji, Papua New Guinea, Australia, Samoa, and Tonga (Ward 1987, O'Dor *et al.* 1993). Nautiluses have limited visual abilities and detect light wavelengths only shorter than 650 nm, with the most efficient absorption occurring at 467 nm (Muntz 1986). They also inhabit a primarily aphotic environment and are commonly found at depths of 150-300 m. Because the internal environment of their shell is resistant to pressure change, nautiluses dwell in depths up to 803 m before shell implosion occurs (Saunders and Landman 1987, Jordan *et al.* 1988).

Nautiluses are slow moving and non-visual, and in general their life history strategies differ greatly from their highly visual relatives, octopuses, squids, and cuttlefish (subclass Coleoidea), which typically live at shallower depths although not exclusively. Aside from life-history strategies,

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[1] Present Address: 333 East 102nd Street, Suite 726, New York, New York 10029, U.S.A.

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* From the symposium "Cephalopods: A behavioral perspective" presented at the joint meeting of the American Malacological Society and Western Society of Malacologists, held 29 July to 3 August 2006 in Seattle, Washington.

nautiloids and coleoids differ in external morphology as well. Coleoids typically possess 8-10 appendages (arms and/or tentacles), all of which are lined with mechanoreceptors and chemoreceptors with the latter occurring particularly within the suckers (Hanlon and Messenger 1996, Cheng and Caldwell 2000, Messenger 2001). Nautiloids have 90-94 tentacles that are typically covered with mechanoreceptor and chemosensory cells (Hamada *et al.* 1978, Fukada 1987, Ruth *et al.* 2002). Nautiloids also have a gas-filled external shell that is sectioned into chambers. Coleoids possess highly developed eyes with lenses that form distinct images. The eyes of *Nautilus* lack a lens but are capable of forming images and capturing light in dark environments, including bioluminescence (Muntz 1994a, 1994b). Given the vast ecological and morphological differences between coleoids and nautiloids, it is a reasonable prediction that each group would use sensory systems, such as vibration detection, differently.

Sources of sound in the ocean include seismic activity, storm events, man-made contributions, and biological activity. For an animal to identify sound as a stimulus, it must extract a signal from the ambient sound environment or, more informally, from background noise (Rogers and Cox 1988). Sound emission can originate from many different sources, but all sound production begins in a similar fashion: a longitudinal, propagating mechanical wave is generated by a change in volume, physical oscillation, or movement. Disturbances from a change in volume that originate from a single pole, such as a pulsating sphere or the inflation of a teleost swim bladder, are referred to as monopole sources. Dipole sources result from a disturbance in the medium in which the volume of the source remains constant but the signal has two points of origin. Typical examples of dipole sources are spheres that vibrate between two points or the sinusoidal movements of a fish moving through the water column (Kalmijn 1988, Coombs 1994).

The acoustic fields created by these sources can be divided into two components: near-field (or local-flow field) and far-field. Stimuli associated with local-flow fields are dominated by particle velocity, displacement, and acceleration, whereas stimuli associated with the far-field can be more accurately measured in scalar quantities such as pressure and density that reflect only the magnitude of the signal. Non-pelagic animals that live in ocean bottoms, coral reefs, intertidal areas, etc., operate primarily in the local-flow field simply because sound waves do not have adequate space to radiate from the source. Pelagic animals frequently operate within both fields and have sensory systems adapted for detection within each field that are dependent on their spatial location at any given time (Bleckmann 1994). An example of the latter would be fishes that possess both lateral-line systems and otoliths, which serve as overlapping sensory systems. The lateral line detects low-frequency stimuli within only a few body lengths of the source, whereas the otolith organs and other components of the inner ear respond to acoustic reception from the outer reaches of the local-flow field well into the far-field (Kalmijn 1988, Braun *et al.* 2002). A similar model could be applied to nautiluses. A plausible mechanism might be that the immediate source (*i.e.*, a group of snapping shrimp) could be detected through mechanoreceptors located on certain tentacles (Ruth *et al.* 2002) while the progression of the wave through the remainder of the near-field into the far-field could be detected by equilibrium receptor organs such as statocysts (Budelmann 1988, Rogers and Cox 1988, Neumeister and Budelmann 1997).

Williamson (1988) tested vibration sensitivity in the northern octopus *Eledone cirrosa* (Lamarck, 1798) and determined that the hair-cell sensitivity within the statocyst of the octopus was three or four orders of magnitude less sensitive than what average fishes can detect. The statocyst of *E. cirrosa* is therefore not considered to be an auditory organ compared to the auditory or far-field detection systems of fishes, although its threshold sensitivities were similar to those of other aquatic invertebrates. More importantly, these results demonstrated that this organ is sensitive to biologically relevant vibrations. Additional studies have suggested that less sensitive vibration thresholds may enhance coleoid survival by lessening the effect of intense acoustic emissions that odontocete predators use to disorient their prey (Moynihan 1985) and that vibration sensitivity need not be confined to the statocyst, indicating that certain mechanoreceptors may be sensitive to vibration as well (Williamson 1988).

It is this line of logic that suggests that *Nautilus* may detect underwater vibration. The statocysts of nautiluses are more primitive than those of coleoids. Perhaps the extreme external morphological differentiation between nautiluses and coleoids has prevented the evolution of such a complex organ due to space or phylogenetic constraints. Additionally, and perhaps more acoustically relevant, there is the gas-filled external shell of the chambered nautilus. Although this shell and its chambers are thought primarily to compensate for buoyancy, principles of underwater acoustics dictate that the shell may also double as a resonating chamber, thereby potentially nullifying the need for the development of a more complex receptor organ.

MATERIALS AND METHODS

Animals

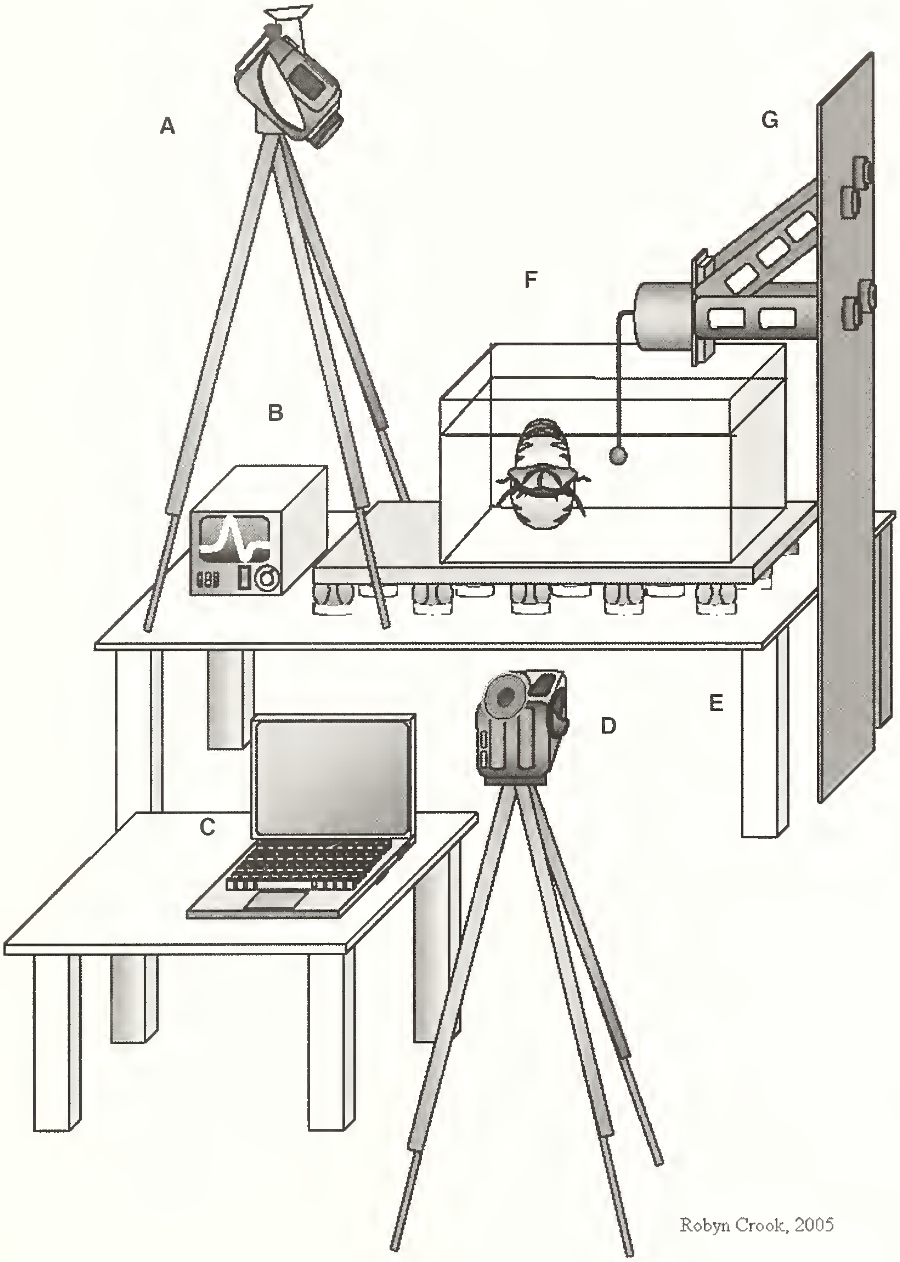
Eleven wild-caught, adult individuals of *Nautilus pompilius*, originally collected in the Philippines and purchased through *Sea-Dwelling Creatures*™, California, were housed in a re-circulating system at the Aquatic Research and Environmental Assessment Center (AREAC) at Brooklyn College of the City University of New York. The animals were divided into two groups and kept separately in a closed system that consisted of two 530-L polyethylene tanks filled with artificial sea water (Instant Ocean™). Both tanks were connected in tandem to a 94.8-L biofilter that contained aeration and filtration media. The animals were kept at constant temperature of 17 °C and at salinities between 32 and 34 psu. Tilapia fish heads (*Oreochromus niloticus eduardianus*) were used as a primary food source, and rations were administered every third day. Daily checks of water quality (temperature, salinity, dissolved oxygen, pH, calcium, alkalinity, ammonia, nitrite, nitrate, and phosphate) were conducted to monitor the system and maintain the health of the animals. Trace elements in the form of a calcium/alkalinity liquid buffer system (B-Ionic™) were added on a weekly basis.

Small and large source-displacement experiments

Experimental apparatus

In two source-displacement experiments (Small Source-Displacement Experiment [SSDE] and Large Source-Displacement Experiment [LSDE]), the experimental arena was a rectangular Plexiglas™ tank (51 cm long × 25.4 cm wide × 31.7 cm tall), containing ~30 cm standing water (Fig. 1). To control for ambient background noise, an insulated and isolated basement room was selected to run the trials. Within the room, the tank was placed on a vibration-absorption table constructed from a granite slab (151 cm × 56 cm × 3 cm). The slab was placed on 12 tennis balls that were separately set in plastic rings and spaced evenly across a metal desk (73.5 cm × 77 cm × 115 cm).

Two digital cameras (Sony Digital Handycam, model DCR-VX1000) mounted on tripods recorded each trial and provided both top and side views. One camera was positioned 1.5 m in front of the long-axis of the tank and the other was placed 1 m above the tank. Visual contact between animals and observers and inadvertent cuing was prevented by placing a removable blind along three sides of the tank and maintaining a minimal distance of 3 m from the uncovered portion of the tank. One fluorescent light bulb was used overhead to illuminate the apparatus, and experimenters did not move in front of the apparatus during the trials.



Robyn Crook, 2005

Figure 1. Experimental setup for source-displacement experiments. A, top-view camera; B, oscilloscope; C, laptop computer; D, side-view camera; E, vibration absorption table; F, experimental tank with animal; G, wall mount with mini-shaker and shaft/bead.

Vibrating stimulus

A dipole source was created by mounting a spherical acrylic bead (18.95 mm in SSDE and 9.44 mm in LSDE) to an aluminum shaft (17 cm in length and 2 mm in diameter) that was bent at a 90° angle and attached to a mini-shaker (Bruël and Kjaer vibration exciter, model 4810). The mini-shaker was fixed to a wall-mounted frame and positioned inside of the tank, such that the bead was located in the middle. Pulse trains were delivered using a laptop computer, and signal outputs were monitored with an oscilloscope (Tenma, model 72-320). Displacement values were based on existing literature (Williamson 1988, Klages *et al.* 2002) and divided into two overlapping ranges that were presented in separate experiments. This format was chosen to minimize habituation to the stimulus and to prevent stress resulting from extended trial times necessary to present the entire range of displacements. The smaller values were tested in the SSDE and ranged from 0.01 to 0.13 mm, whereas the larger values were tested in the LSDE and ranged from 0.08 to 1.12 mm. For the Large Source-Displacement Experiment, a stereo receiver (Kenwood, model VR-615) was used to amplify the signal, thereby increasing the source displacement.

Stimulus signals were created using SigGenRP v.4.4 stimulus design software from Tucker-Davis Technologies. Stimulus presentations were compiled and edited using CoolEdit Pro v.2.1 from Syntrillium Software Corporation recently renamed Adobe Audition v.1.5. Each of the stimulus pulse trains was 5 s long and included ten 2-ms clicks of the same amplitude, separated by nine 0.553-s intervals of silence. Clicks are defined as short, intense bursts of energy that encompass a wide range of frequencies. Stimulus pulses and their respective source-displacements were measured and calibrated prior to the experiment using a Metrolight laser micrometer (model Alpha XO3). All pulse trains were presented only once in each of the trial sequences. Their presentation orders were determined using a random number generator.

Experimental procedures

Trials were conducted on separate days between the hours of 1100 and 1800. The experimental tank was filled with conditioned seawater from the home tank to ensure that each animal was constantly exposed to uniform and familiar olfactory cues. Seven animals were used in the SSDE and five animals were used in the LSDE, three of which were the same (repeated-measures within-subject design; Myers and Well 2003). Animals were transported from the home tank in covered buckets, gently transferred to the test arena, and allowed to habituate for 10 min prior to the start of experimental trials. Following habituation, video recording commenced and individuals were subjected to a 5-min control period during which time no vibrational pulses were administered. The control period was followed by a 5-min "stimulus package" that began with 20 s of baseline silence and continued with the presentation of 11 randomly ordered pulse trains that were separated by 20 s of silence.

Treatment order (control first, stimulus second) was not altered between trials because it was unclear how long the effect of the stimulus on the behavior of the animals, if any, would last. If the stimuli were to be presented before the control in these initial experiments, any continuing effect on the behavior of the animals would reduce the legitimacy of the control data. After trial completion, video recording was stopped and animals were returned to their home tank. The test aquarium was rinsed thoroughly between trials with fresh water to remove any residual individual olfactory cues.

Frequency-sensitivity experiment

Experimental apparatus

The experimental arena was similar to that of the SSDE and LSDE with the exception that a smaller, rectangular Plexiglas™ tank (41 cm × 21 cm × 26.8 cm) containing ~25 cm standing water was used. Additionally, four foam pads that measured 14.5 cm in height were used to absorb background vibration, and only one camera, placed 1.5 m in front of the long axis of the tank, was used.

Vibrating stimulus

Stimulus frequencies were generated in an identical fashion to that described previously in the SSDE section. Stimulus presentations were compiled and edited using CoolEdit Pro v.2.1 from Syntrillium Software Corporation (Adobe Audition v.1.5). The 5-min stimulus package consisted of 11 randomly ordered frequencies (10, 50, 75, 100, 150, 200, 300, 400, 500, 750, and 1000 Hz) that were chosen based on existing literature and by determining which frequencies might be most prevalent in the animal's natural habitat (Williamson 1988, Klages *et al.* 2002). A 0.37 mm bead displacement was used for all frequencies so corresponding source-velocities could later be determined. This value was chosen based on results from the LSDE that revealed that this displacement value caused a large decrease in nautilus ventilation rate and was large enough to eliminate concerns of background interference. Each frequency emission was 5 s long and was separated by 20 s of silence. A selected frequency was included only once per trial sequence and the presentation orders of the frequencies were determined with a random number generator.

Experimental procedures

See *Experimental procedures* from the previous experiment for habituation procedures. Eight animals were used in the frequency-sensitivity experiment (FSE), and trials consisted of a 5-min control period (silence) and a 5-min stimulus-set presentation consisting of 11 randomly ordered frequencies. The presentation of the treatment category (control or stimulus) was alternated between trials, and a 5-min buffer period (silence) was inserted between treatments to control for order effects.

Data collection and behavioral analysis

Data were collected from the video recordings by two independent "blind" observers using a Sony DHR-1000 digital video-cassette recorder. A suite of five typical *Nautilus* behaviors (Basil *et al.* 2005) was identified prior to the experiment but no *a priori* assumptions were made about whether those behaviors would be evident or about their magnitude and polarity. Trials were subdivided into 5-s bins and individual behavioral measurements were recorded in real time for each bin. Typical behaviors such as rocking, touching the bottom of the tank (not just resting on the bottom), tentacle extension (expressed as a percentage of body length), and the "cat's whiskers" foraging posture were not detected in any of the trials. Ventilation rate was a consistent and robust measure of response and has been used as an experimental measure for other cephalopods (King and Adamo 2006) and, hence, will be the focus of all our analyses.

Ventilation rate was defined as the number of completed respirations per 5-s interval and is abbreviated as ventilation rate/5s or VR. This behavior was recorded by observing the area of the mantle cavity bilaterally located posterior to the eye or by minor vertical oscillations of the entire animal produced by water expulsion through the hyponome (Fig. 2). A completed respiration was defined as either (1) the period between one closure of the mantle to the next or (2) the deviation in movement of the animal from a standing position to a position either slightly above or below, and then the return to the initial standing position, which has proven to be another reliable indicator of ventilation in these animals (Basil *et al.* 2005).

Statistical analysis

A repeated-measures within-subject design was used for all three experiments (Myers and Well 2003). Paired samples Student's *t*-tests were used to compare ventilation rates of animals between treatments to determine if exposure to a vibratory stimulus had any effect on behavior. Both control and stimulus periods were 5-min long and data were collected in 5-s intervals or time bins. Data for each time bin were combined and averaged for each treatment and for each animal.

Additional analyses were then performed on data that were divided into categories based on the spatial and temporal response of the animals. Two "distance" categories were created: responses of animals <20 cm and >20 cm from the source. Spearman's Rank correlation tests were used to examine the correlation between distance from the source and ventilatory behavior. In instances where the same animal was used in more than one experiment, a single mean ventilation rate was used to prevent pseudoreplication. This was not possible for analyses that examined potential effects of distance from the source on ventilation rate, as animals that participated in more than one experiment often occupied both distance categories, therefore requiring that the trial averages be separated for analysis.

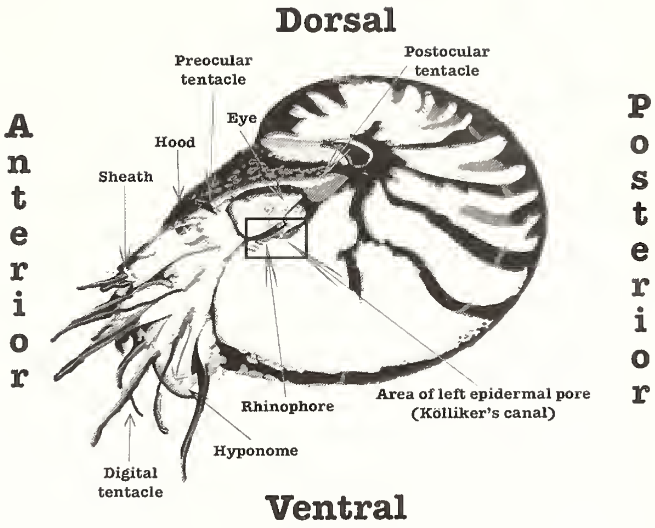


Figure 2. Lateral view of *Nautilus pompilius pompilius*, depicting various external components with emphasis on the location (near the rhinophore and the epidermal pore that connects Kölliker's canal with the left statocyst) of the mantle cavity that was used to count ventilation rates.

To describe the reaction of the animals through time, five temporal categories were created by subdividing the stimulus category. During each trial, a maximum of 11 data points were collected for each of the following stimulus categories: 5-s stimulus presentation (5 s stim), 1-5-s post-stimulus (1-5 s post), 6-10-s post-stimulus (6-10 s post), 11-15-s post-stimulus (11-15 s post), and 16-20-s post-stimulus (16-20 s post). Categorical averages for each trial, and subsequently each animal, were obtained and paired-samples *t*-tests were used to compare control data to each of the 5-s post-stimulus categories.

As an additional note, mean ventilation rates varied greatly between animals so numerical ventilation rates were converted into percentage change from the control to demonstrate changes in behavior graphically. However, all statistical tests were performed on the actual ventilation values as opposed to the percentage values to avoid an artificial increase or decrease in probability due to the imposition of fixed limits (0-100) on the measure.

RESULTS

Overall combined results for all experiments

Twenty trials using 11 animals were conducted. A significant decrease of 8.23% in ventilation rate/5 s occurred between control and stimulus treatments across all animals (Paired-Samples Student's t-test, $N = 11$, $t = 2.61$, $P = 0.03$) with a mean control VR of 4.06, $SD = 1.72$ and a mean VR in the presence of a stimulus of 3.70, $SD = 1.45$.

Mean ventilation rates for *Nautilus* remained below control values for at least 20 s post-stimulus presentation (Fig. 3). Paired-Samples t-tests revealed that the largest decrease of 9.9% was observed during the actual 5-s stimulus presentation (t-test, $N = 11$, $t = 2.90$, $P = 0.02$) and the smallest decrease of 6.9% occurred 5 seconds after that (t-test, $N = 11$, $t = 2.37$, $P = 0.04$). The responses of animals in the remaining three 5-s post-stimulus bins were 8.6% lower than controls in the 6-10 s post-stimulus bin (t-test, $N = 11$, $t = 2.80$, $P = 0.02$), 7.4% lower during the 11-15 s post-stimulus bin (t-test, $N = 11$, $t = 2.26$, $P = 0.048$), and lastly 8.2% lower than controls during the 16-20 s post-stimulus bin (t-test, $N = 11$, $t = 2.26$, $P = 0.05$), respectively.

Data from 15 trials using eight stationary animals were examined to determine if ventilation rate decreases in *Nautilus* when animals are closer to a vibrating stimulus. Only animals that remained stationary throughout the trial were used so their distance from the source would be constant. Five of the animals participated in more than one trial and, unless an animal produced values for both distance categories, their mean VR values were averaged between trials and used in the analysis. Six animals <20 cm from the source had an average of VR 2.83, $SD = 1.07$ whereas six animals that were >20 cm demonstrated a slightly higher average VR of 2.87, $SD = 0.38$. No significant correlation between distance from the source and VR was found (Spearman's Rank correlation, $N = 8$, $r_s = 0.22$, $P = 0.60$). Additionally, a subset of animals was selected for which data existed in both distance categories for each animal. Means from both categories were compared to determine if distance from the source caused significant differences in VR. Although no significant differences were evident (Paired-Samples t-test, $N = 4$, $t = -2.52$, $P = 0.09$), animals vented at a rate that was 8.0% lower when they were closer to the stimulus than when they were >20 cm from the origin of the vibrations.

When source-displacement increased, animals exhibited a decrease in their ventilation. Pearson correlations examined ventilation rates in seven animals from the SSDE and LSDE (Fig. 4) across nine trials. Three animals were <20 cm from the source and six animals were >20 cm from the source. A significant inverse correlation was found between source-displacement and VR for animals that were <20 cm from the source (Pearson correlation, $N = 6$, $r = -0.57$, $P = 0.01$). No significant correlation was found between source-displacement and VR for animals that were >20 cm from the source (Pearson correlation, $N = 3$, $r = 0.43$, $P = 0.06$). On average, animals from the SSDE and LSDE, when exposed to a vibratory stimulus, ventilated at a rate that was 11.72% less than the control VR when they were <20 cm from the source, compared to a 5.38% decrease for those that were >20 cm from the source.

When source-velocity increased, as seen during the FSE, animals also exhibited a decrease in their ventilation. Mean ventilation rates for five animals which were used in the FSE were examined across 12 source-velocity categories (Fig. 5). Four animals were <20 cm from the source and the remaining animal maintained a distance >20 cm from the source. No significant relationship was found to exist between source-velocity and VR

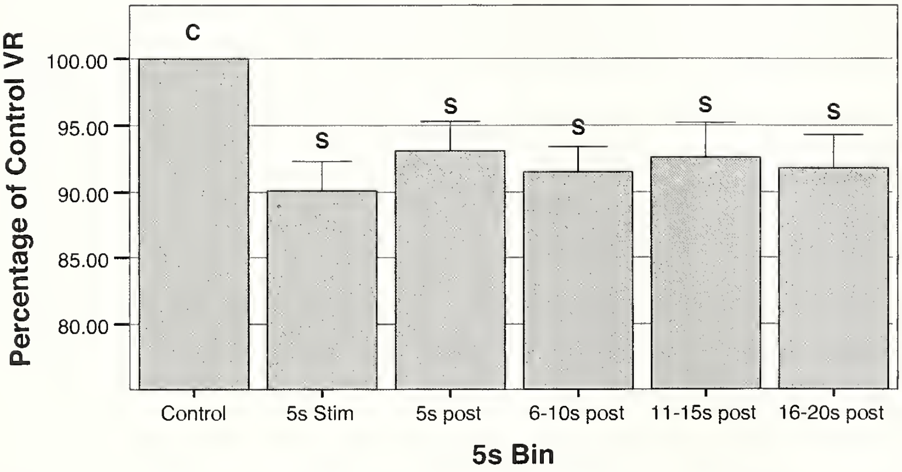


Figure 3. Bar graph depicts the mean percent change in ventilation rate (VR) of the control when compared to each of the five stimulus and post-stimulus time categories. Each bar labeled "S" represents a 5-s period of time that begins with the presentation of the stimuli and continues for a 20-s post-stimuli period. The bar labeled "C" represents a 5-min control period. Significant decreases between the control and stimuli bins were found for each of the five time categories but no continual decrease in VR over time was observed. Error bars show +1 *SE*.

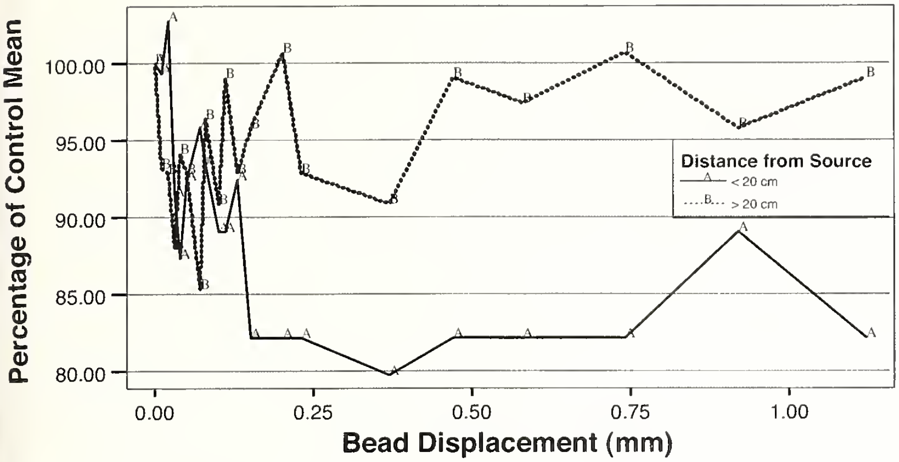


Figure 4. The impact that animal distance from the source and source-displacement has on ventilation rate. Data shown are from two experiments, the Small Source-Displacement Experiment (SSDE) and the Large Source-Displacement Experiment (LSDE), and account for eight animals across nine trials. Three animals were <20 cm and six animals were >20 cm. Bead displacement refers to the distance traveled by the leading edge of the bead and does not include bead diameter.

for animals that were <20 cm from the source (Pearson correlation, $N = 4$, $r = -0.52$, $P = 0.08$). No statistical correlation could be conducted between source-displacement and VR for animals that were >20 cm from the source because of an inadequate sample size ($N = 1$). Animals from the FSE, when exposed to a vibratory stimulus, ventilated at a rate that was 16.3% less than the control VR when they were <20 cm from the source compared to a 0.6% increase for those that were >20 cm from the source.

Additionally, treatment-order effects and the possibility of habituation across trials in nautiluses were examined. The analysis of presentation order, control first or stimulus first, revealed that no treatment-order effect was evident in the FSE (Independent Samples *t*-test, $N = 8$, $t = 1.55$, $P = 1.44$).

DISCUSSION

The major finding revealed by these experiments is that *Nautilus* responds to underwater vibrations. Animals almost always reduce their ventilation rate in the presence of a vibratory stimulus: there were significant decreases in ventilation rate during a majority of trials when the animal was exposed to vibratory stimuli.

Comparatively speaking, these findings are relevant to research conducted previously on other invertebrates, such as Williamson's (1988) investigation into the vibrational sensitivity of the statocyst in the northern octopus where a minimum particle-displacement threshold of 0.12 μm was determined and the study conducted by Klages *et al.* (2002) that noted that the deep-water amphipod *Eurythenes gryllus* produced particle displacements of 0.05-0.3 μm between 70 and 200 Hz when feeding and swimming. This work has demonstrated that nautiluses are capable of responding well within these ranges of displacements and frequencies, so future work should focus on determining practical applications of this system in the wild. The detection of signals in the wild can benefit *Nautilus* in many ways. A decrease in ventilation rate could possibly serve as a mechanism for predator avoidance. Similar responses have been observed across multiple groups of animals including cephalopods. King and Adamo (2006) demonstrated that the cuttlefish *Sepia officinalis* Linnaeus, 1758 reduced ventilation and

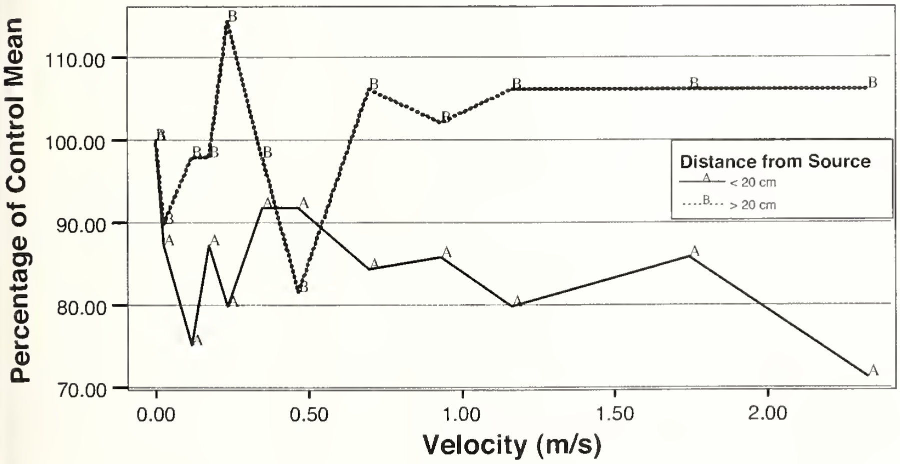


Figure 5. The impact that animal distance from the source and source velocity have on ventilation rate. Data shown are from the Frequency-Sensitivity Experiment (FSE) and account for five animals across five trials. Four animals were <20 cm from the source and one animal was >20 cm from the source. Velocity represents varying source-intensities that were presented randomly.

cardiac rates when exposed to sudden visual stimuli, in preparation for a flight response. Additionally, the authors identified four hypotheses in the literature that offered explanations for this behavior, one of which was that animals decrease ventilation to increase crypsis. Although they rejected this hypothesis, suggesting that cuttlefish decrease VR in preparation of a flight response, the hypothesis can be applied to nautiloids since no movements associated with the stimulus were observed during experiments.

From a biological standpoint, decreasing respiratory rates may serve as a defense mechanism. Presumably, approaching predators emit a range of vibratory stimuli resulting from motion, such as the sinusoidal movements of fish. Therefore, such a mechanism would work most effectively in concert with cryptic coloration, by reducing overall rocking movement as the predator nears.

Conversely, decreasing respiration may benefit an animal's predatory success. This is not to imply that nautiluses are formidable hunters—but a sit-and-wait strategy is possible. These animals spend most of their lives associated with coral reefs that are teeming with potential prey items. Perhaps nautiluses, upon detection of certain chemical or vibrational cues, decrease respiration to make themselves less conspicuous to an unsuspecting prey. However, it is improbable that a decrease in VR is an offensive strategy since anecdotal evidence suggests that captive animals increase respiratory activity when exposed to food sources (Soucier, pers. obs.).

Nautiluses likely detect vibration with epithelial tactile receptors on the tentacles, mechanoreceptors below the rhinophore, or some other innervated system. In cuttlefish (Komak *et al.* 2005), epidermal lines along the mantle and arms containing polarized hairs are able to detect local water movements and subsequently integrate that information into behavioral responses. The locations of these potential receptors in *Nautilus* were, however, not ascertained in our experiments. Additionally, the role of the gas-filled external shell acting as a resonating mechanism was not investigated during our experiments but should not be excluded from consideration as a contributing factor.

Irrespective of the mechanism, any additional sensory system that an animal can use, whether it is in conjunction with alternate systems or serving as a primary system would be beneficial to the survival of that animal. Based on the average depth in which these animals live, the nektobenthic niche that they occupy, and the lack of information regarding their feeding and mating strategies, an evolutionary argument could be made for possessing a mechano-sensory system capable of detecting hydrodynamic disturbances and/or substrate-borne vibrations.

In regard to latency of response or time-specific responses, our experiments revealed no temporal trends within our time periods because significant decreases in ventilation rate ranged from the stimulus presentation to the 16-20s post-stimulus period. These animals can respond to the stimulus for up to at least 20 s post-presentation, and the distance from the source and the components of the signal should be the focus of future investigations.

The results of these experiments clearly indicate that *Nautilus pompilius pompilius* can detect and respond to vibrational stimuli. To what end this sensory system serves, whether it is mate selection, prey acquisition, predator avoidance, or a combination of multiple evolutionary functions, has yet to be determined. What has been established is that the recognition of these signals and subsequent behavioral response may pose some type of evolutionary advantage.

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Octopus sucker-arm coordination in grasping and manipulation*

Frank W. Grasso

Department of Psychology, Brooklyn College, City University of New York, 2900 Bedford Avenue, Brooklyn, New York 11210, U.S.A., FGrasso@brooklyn.cuny.edu

Abstract: In natural settings octopuses use their arms and suckers in a variety of dexterous manipulation tasks, such as extracting prey from crevices and burrows, opening bivalve shells, and arranging middens in front of den entrances. Octopuses use multiple suckers on a single surface for a power grasp that supports their locomotion or permits the animal to carry or move small objects. Similar to squids engaged in prey capture, octopuses can project an arm from their body, attach a group of distal suckers, and pull an object toward themselves by shortening the arm. I investigated octopuses' use of suckers in similar tasks under controlled, reproducible laboratory conditions. Because larger suckers can generate larger adhesion forces, I hypothesized that the larger suckers toward the base of the arm would be preferred in tasks requiring the arm to employ greater forces. Octopuses did not use the strategy found in squid tentacles: applying suckers of appropriate force generation to a surface and lifting or pulling the arm. Instead, in many cases they used a variety of arm movements in combination with different functional groups of suckers. In addition, different arms performed different roles. When animals were restricted to the use of a single arm, they preferred to use suckers in the middle positions of the arm to support this coordinated arm-sucker activity. Contrary to a view of suckers as passive agents reflexively reacting to surface contact, these results are consistent with the known neural organization of the octopus arm and also with complex sucker-arm coordination in the performance of manipulation tasks.

Key words: Octopus, grasping-behavior, suckers, coordination

> *Octopuses move in a mysterious way. Being flexible, the movements that they make are often difficult to specify and correspondingly difficult to investigate. The literature does not contain a description of octopod walking comparable with descriptions of the six-legged, tripod gait of insects or the stereotyped locomotor patterns of snails or polychaetes. Descriptions of posture run into very similar difficulties and perhaps partly because of this, research on motor control in cephalopods has proved a less attractive proposition than research on sensory analysis and learning.* (Wells 1978: 246)

Wells' claim that studies of motor systems in cephalopods have lagged behind those of other sensory and learning systems still rings true today for studies of *Octopus* Cuvier, 1797 and for the reasons he cites. Progress has been made with kinematic descriptions of reaching and fetching behavior that have inspired neural and physiological models of arm control in these activities (Gutfreund *et al.* 1996, 1998, Matzner *et al.* 2000, Sumbre *et al.* 2001, 2005, 2006) and a systematic description of the movements of *Octopus* arms has also been developed (Mather 1998). The muscular-hydrostat mechanisms by which arm movements are effected have provided a conceptual framework for understanding limb movement and manipulation in the absence of hard parts (Kier and Smith 1985). In addition, researchers have begun to explore and explain the neurophysiology of bend generation in the arm (Gutfreund *et al.* 1998, Matzner *et al.* 2000, Sumbre *et al.* 2001). However, since Wells (Wells and Wells 1957a, 1957b, Wells 1978), little attention has been directed toward the behavioral repertoire involving the suckers on the arm which provide the octopus with contact tactile and chemosensory information and fine local manipulation.

The arms of octopuses can bring suckers into a position to sense or grasp a surface of interest to the animal. Though the flexibility of their arms makes them quite capable of it, octopuses are rarely observed to wrap their arms to grasp objects. The method octopuses employ in securing purchase on objects varies with the object and context; the wrapping often appears to be a natural continuation of arm momentum following abrupt contact with a fixed object. The suckers are integral to much of the directed behavior of octopuses; Yet, apart from some excellent quantitative studies of their adhesion mechanism (Smith 1991, 1996), their mode of action has received little attention. This report begins to fill this gap by analysis of simultaneous observations of arm movements and the actions of scores of suckers under natural and experimental conditions.

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* From the symposium "Cephalopods: A behavioral perspective" presented at the joint meeting of the American Malacological Society and Western Society of Malacologists, held 29 July to 3 August 2006 in Seattle, Washington.

The extent to which sucker and arm movements are coordinated or independent is currently unknown. Hanlon and Messenger (1996: 15) reflected that "In fact the nervous system of the arms, which contains more neurons than the whole central brain (Young 1971), is in some ways curiously divorced from the rest the brain and many of the arms actions are performed without reference to the brain." The same comment applies to the relationship of control between the arms and the suckers: suckers have some degree of autonomy but must move in ways that are not in conflict with ongoing arm activity. Studies have shown that a single octopus arm detached from the rest of the animal retains considerable capability for coherent response to stimuli (Wells and Wells 1957a, Rowell 1963, 1966, Altmann 1971, Wells 1978, Gutfreund *et al.* 2006). Yet, all eight arms are not completely independent as is clear because the animal is capable of coordinating all its arms, and arm preferences exist (Byrne *et al.* 2006). These studies have focused on the actions of the arms and not the contributions that the suckers, the primary contact sensing and local action organs, make.

The suckers, too, have the appropriate (direct or indirect) neural connections (Fig. 1A) to send information to and receive information from the brain and the arm on which they are situated (Graziadei 1971). Each sucker has a committed local ganglion. This ganglion receives an enormous number of afferent fibers: chemosensory and mechanosensory axons from the sucker rim as well as proprioreceptors (muscle sense) from the various muscles of the sucker. These fibers pass through the nerve connecting the sucker and the ganglion of the sucker (Fig. 1A). This nerve also carries motor neuron axons to control the sucker muscles. The ganglion also carries on bidirectional communication with the main nerve cord of the arm, the chain of brachial ganglia. This communication is carried via the nerve connecting the sucker ganglion to the brachial ganglion (Fig. 1) and not much is known about its function. The brachial ganglion is one of a chain of ganglia which enlarge, increasing their neuron counts and neurpil volume directly over each sucker. Anatomically, they form a chain of intercommunicating ganglia along the length of the arm, each of which appears to be intimately involved in sucker information processing. Finally, these ganglia make direct connections of their own to the sucker, bypassing the sucker ganglion, through the nerve connecting the brachial ganglion and the sucker (Fig. 1). This nerve carries sensory fibers

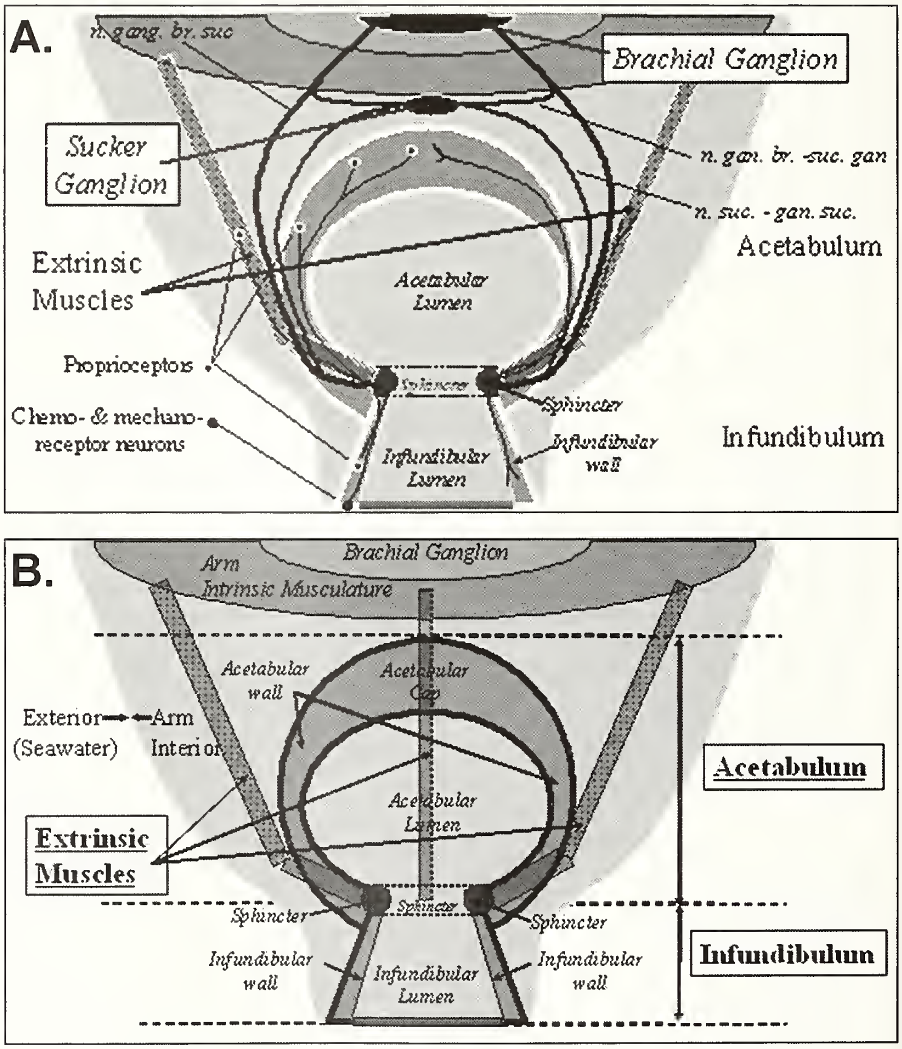
A.
n. gang. br. suc
Brachial Ganglion
Sucker Ganglion
n. gan. br. -suc. gan
n. suc. - gan. suc.
Acetabulum
Extrinsic Muscles
Acetabular Lumen
Proprioceptors
Sphincter
Sphincter
Chemo- & mechano-receptor neurons
Infundibular Lumen
Infundibular wall
Infundibulum
B.
Brachial Ganglion
Arm Intrinsic Musculature
Acetabular wall
Acetabular Cap
Exterior (Seawater)
Arm Interior
Acetabulum
Acetabular Lumen
Extrinsic Muscles
Sphincter
Sphincter
Sphincter
Infundibular wall
Infundibular Lumen
Infundibular wall
Infundibulum

Figure 1. Schematic diagrams of a typical octopus sucker and arm attachment in cross-section perpendicular to the long axis of the arm. A, The functional divisions described in the text for adhesion generation. B, The gross neuroanatomical connectivity of those functional parts in relation to the arm. Abbreviations follow those used in Young (1971): n.suc.-gan.suc., nerve connecting the sucker and the ganglion of the sucker; n.gang.br.-suc.gan., nerve connecting the sucker ganglion to the brachial ganglion; n.gang.br.-suc., nerve connecting the brachial ganglion and the sucker; n.gan.br.-gan.suc., nerve running from the brachial ganglia to the sucker ganglion.

from the sucker and possibly motor fibers to the sucker. This brief sketch of the neuroanatomy demonstrates that the connections exist for rich information exchange between the suckers and the arm chain ganglia and through the brachial ganglia indirectly between the suckers and the brain. The functional roles of these identified pathways have yet to be studied.

In squid tentacles the roles of sucker and tentacle have been studied behaviorally and kinematically and the control of the suckers appears to be much simpler. From these studies it appears that the coordination of limb and sucker action is a passive and not an active one. The squid (*Loligo pealei* Lesueur, 1821) combines the actions of its paired tentacles and suckers in prey capture (Kier 1982, Van Leeuwen and Kier 1997). The terminal club of the tentacle, covered with suckers, is ballistically propelled toward the squid's prey in <300 ms. The process is too fast for tentacle-sucker coordination, so a local reflex-triggered by mechanical contact, in turn triggers rapid sucker attachment. It is possible, despite the anatomical connections described above, that the actions of octopus suckers follows a similar plan where the movements of the arm bring the sucker into contact with some surface, and that surface contact in turn triggers a reflexive sucker attachment. Though there is evidence that this is not always the case (Wells and Wells 1957a, 1957b, Rowell 1963, 1966), the idea that suckers are triggered to attach by mechanical contact is a parsimonious explanation for sucker operation in octopuses that cannot be ruled out in all situations.

Anatomical organization of octopus suckers, which differs in sophistication from those of the squid, indicates that octopus suckers are well suited to support active coordination. The club suckers on the squid tentacle are composed of a single chamber surmounted by a large internal muscle which acts like a piston to develop a negative pressure for adhesion in a few milliseconds (Van Leuven and Kier 1997). Octopus suckers are two-chambered, radically-symmetric structures (infundibulum and acetabulum) suspended from the oral surface of the octopus arm that incompletely enclose a volume of the surrounding seawater (Fig. 1B). Like squid suckers, they act on ambient seawater to reversibly attach an object to the octopus arm or the octopus arm to a fixed surface with which the sucker makes contact. The mechanisms by which they facilitate grasping in octopuses have been inferred from anatomy (Kier and Smith 2002). They have an elegant division of function that is absent in the squid: the muscles of the infindibulum reshape the sucker rim to conform to the exterior surface; after a seal is formed (completing the enclosure of the volume), the muscles of the acetabulum expand its internal volume to produce negative pressure (and therefore adhesion force). The major difference between squid tentacle and octopus-arm suckers resides with the third functional group of muscles in the sucker. The extrinsic muscles of each sucker attach at the junction of the infundibulum and acetabulum and on the arm itself. With the surface held, the extrinsic muscles are arranged to act antagonistically to rotate the sucker rim in virtually any plane around the long axis of the arm, along with whatever it is attached to. It is these extrinsic muscles which suggest octopus suckers evolved to support a *manipulation* as well as *attachment* function.

In addition to this motor function, octopus suckers appear to play an important sensory role. The surface of the octopus arm is studded with mechano- and chemoreceptors but their density is extremely high in the sucker rim: on the order of 10^4 per sucker (Graziadei 1964, Graziadei and Gagne 1976). As mentioned above, these receptors, along with anatomically identified proproiceptors in the sucker, project their axons to make synapses in the small ganglion that lies over each sucker and in the brachial ganglion of the axial nerve cord that runs the length of each arm (Fig. 1B) (Graziadei 1965, 1971). Thus, the observed interconnectivity of the suckers and arm ganglia serve a primarily sensory function rather than a motor control. The receptor and neural organization agree with observations of complex motions made by suckers engaged in apparent sensory exploration. Suckers in an otherwise stationary arm are occasionally observed to reshape themselves by extension, retraction, and rotation to follow surface contours and edges with only the rim in contact and without forming a seal and sucker attachment. These movements occur outside the octopus's field of the vision and therefore appear to require local sensory feedback and motor integration.

The studies reported here sought evidence of active arm-sucker coordination in two forms. First, correlations between arm and sucker activity during spontaneous behavior of freely moving animals were studied. Patterns of activity in groups of suckers that varied with the behavior of the octopus as a whole entity would be consistent with active coordination. Second, I experimentally manipulated the force required to complete a task, thus requiring the octopus employ a different mechanical approach. The adhesive force of suckers is proportional to their size (Smith 1991, 1996) and suckers on any given arm become smaller in size distally (Voight 1993). Thus, if the octopus adjusted the use of its suckers depending upon the force required for a given task, this would provide evidence that some feedback about the appropriate force level was shared between the suckers and the arm or between the suckers and the brain.

MATERIALS AND METHODS

Natural observations of sucker use

Animals

Four wild-caught adult *Octopus bimaculoides* Pickford and McConnaughey, 1949 were filmed in their home tank *ad* *libitum* to capture examples of their use of suckers on one vertical glass wall. These animals had arms that were approx. 15-20 cm long at the time of the experiment. I did not determine the sex of these animals. However, one of them showed somewhat enlarged suckers toward the base of its second arms, indicating that this animal was male. The animals were fed *ad libitum* on a diet of clam meat, frozen shrimp, and, occasionally, live crabs. These octopuses were different individuals from those studied in the following experiment.

Video acquisition

Animals were filmed at 30 fps using a JVC MiniDV digital video camera (GR-D250U) positioned outside the tank to encompass the entire pane (resolution ≈1 mm per pixel). The tanks were standard 113.59-L tanks of dimensions 76.2 × 53.34 × 33.02 cm. The animals had lived in these tanks for at least 3 weeks and were therefore habituated to the tanks and the conditions in the room where they were housed. Laboratory personnel were absent from the room or visually isolated from the animals by a curtain while the footage was collected. Approx. four hours of this footage was surveyed for periods during which (1) single arms could be observed when (2) 20 or more contiguous suckers were continuously visible for (3) 10-30 seconds. Several sections of video were obtained that I refer to here as “continuous video segments.”

Scoring the video

These sections were scored at 1-s intervals. When suckers are attached to a glass surface they assume a characteristic appearance: they are flattened and look like enlarged white discs compared to their unattached state, and the sphincter is clearly visible and round. An observer scored each sucker as “Free” (F), “In-Contact” with the surface (C), “Partially Attached” (P), or “Attached” (A) for each second in each series. Suckers scored as F had no part of the sucker in contact with the glass; C had less than the whole sucker rim in contact with the glass; P had the entire rim but not the sucker sphincter in contact with the glass; and A the entire rim and sphincter were in contact with the glass – the sucker was flat and its radius enlarged. These categories were easy to distinguish. Inter-observer agreement on these categories for a 30-s section of tape in which 45 suckers were visible was at least 95% for three practiced observers.

Analysis

The attachment data were a series of “sucker states” in time. Plots of these data were made to represent the sequence of sucker attachment down the arm. The scored categories of C and P were grouped in a single category while F and A were retained as separate plot categories. I also computed probabilities that a given sucker and its neighbors would be co-active. For these analyses I assigned each sucker a value of 1 each time it was observed to be in state A and a zero if it were in state F, C, or P. For each time step, I counted the number of coincident attachments for each sucker and its neighbors one, two, three, and more suckers in proximal or distal directions along the arm. With this I had a description of the coincident activation of each sucker with all the observed suckers in a coordinate system centered on the individual sucker. I aligned each individual sucker’s co-activation pattern to this sucker-centered frame and, by adding the coincidences for each distance from the sucker, computed the total number of co-incident attachments for each neighbor. This total, divided by the total number of activations observed at that distance, is the proportion of co-activation, or probability of co-activation, observed in that particular frame of a continuous video segment. Individual traces are shown as time series and averages of the entire series (Figs. 2A-C).

Object raising experiments

Experimental animals

The animals used in this experiment were six wild-caught *Octopus bimaculoides*. I did not determine the sex of these animals, but two of them showed somewhat enlarged suckers toward the base of their second arms, indicating that they were male. They had arms 15-20 cm long. This species possesses 150-300 suckers, including extremely small suckers (<1 mm diameter) at the tip, arranged in two staggered rows (Voight 1993). Octopuses were maintained in individual, transparent Plexiglas-walled chambers in a recirculating, artificial seawater system and were fed *ad libitum* on a diet of clam meat, frozen shrimp and, occasionally, live crabs. This did not appear to affect their motivation for capturing live

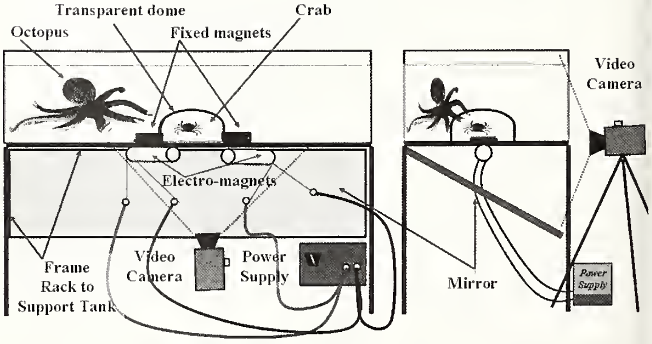

Figure 2. The apparatus used in the dome-raising experiment. The left side of the figure shows a front view of the apparatus and the right, a side-view. The position of the mirror permitted the video capture of a side and bottom view with a single camera. The diagram is schematic and not drawn to scale.

crabs in the testing tank. Animals could see individuals in adjacent chambers but could not make physical contact.

Apparatus

Tests were conducted in a glass-bottomed aquarium (114-L), similar to that used in experiment 1, supplied with continuously refreshed water from the animal's housing system (Fig. 2). A mirror was placed beneath the bottom of the tank at a 45 degree angle so that the octopuses' movements and sucker use could be viewed from below. A single JVC MiniDV digital video camera (GR-D250U) was placed so that half the field of view captured this view from below and half captured the side view of the tank and animal activity. A transparent, ~5-cm diameter glass dome was placed, rim-down, on the floor of the tank. The dome was fitted with two fixed magnets positioned at opposite sides of the rim. These magnets were held to the dome with a single, long cable tie and thermal glue. The magnets were aligned with the positive pole up and the negative down relative to the dome. The fixed magnets' flat surfaces were parallel to the rim of the dome; when the dome was in place, the rim and magnets lay flush with the floor of the tank. Two electromagnets were positioned beneath the tank and aligned with the fixed magnets to provide variable force required to complete the task. Electric current from an Elenco Precision Regulated DC power supply (Model XP-603) was adjusted to modify the strength of the magnetic field they exerted. The force of these activated electromagnets exceeded the force of gravity so that the magnets were held in place. We used a spring scale to determine the force required to detach the dome from the tank floor. We varied the current through the electromagnets over a range of 0 to 1 amperes and recorded the force required to detach the dome at a variety of current levels. A regression analysis of the current supplied to the electro-magnets allowed us to estimate the force required to detach the dome ($F = 3.88\ C + 10.48$; $r^2 = 0.54$). I could produce a 4 N difference in force due to the action of the magnets. The weak correlation led us to use the settings as "weak" or "strong" magnetic force as conditions in our experiments. The weight of the dome and fixed magnets required 10 N to move when the tank was full of water so the range of forces an octopus was required to apply to detach the dome varied between 10 and 14 N.

Trial procedure

At the start of a trial, a crab (~1-2 cm carapace length) was placed under the dome and the electromagnets were activated to produce the desired level of force. In early trials, the octopus was released into the chamber with the dome and was free to move about the tank and use all of its appendages. On later trials, the animal was released on the other side of a partition with a 1.5 cm hole which permitted the animal to reach the dome but limited its appendage use to one or at most two arms. The camera recorded the activity of the animal at 30 fps. My protocol called for the termination of the trial if the octopus did not raise the dome within 30 minutes. This time limit was never reached, and the octopuses always raised the dome a few minutes after being placed in the tank.

Scoring the behavior

We scored the number of suckers attached to the dome at the time the dome was raised. The scoring method was the same as that used in the observations reported above (states F, C, P, A). We also scored the portion of the arm in contact with the dome when the animal first raised the dome. The observer judged whether the proximal, middle, or distal third of the arm was in contact with the dome. The time that the dome was first raised was judged as the video frame just before the electromagnets began to fall.

RESULTS

Natural observations of sucker use

Sucker attachments were sparse in the video footage examined. On average 18.07% ± 13.78 (SD) of the suckers observable (20 to 60, depending on the trial) on a given arm were attached at any given time, and instantaneous values ranged from 0 to 39%. We observed no occasions on which all the suckers on an arm were attached—even when the arm was motionless along its entire extent.

The spatial arrangements and temporal sequences of sucker attachment varied with the activity in which the animal and the arm were engaged. Some spatial patterns of simultaneous sucker attachment and certain temporal sequences of attachment were repeated often in these observations.

Adjacent suckers on opposite sides of an arm were frequently observed to attach to the surface in anti-phase: alternating attached and unattached states. Groups of six, ten, or even 15 adjacent suckers would be involved in these coordinated patterns (see Figs. 1B, 2A). Sometimes this would persist as a single alternation; on other occasions it might go on for several seconds, displaying as many as eight cycles of attachment and release. In such an "arm walk", the arm is moved along the surface of the glass, held in a fixed orientation for several seconds as the suckers advanced in leading and trailing pairs across the tank wall.

Suckers could also hold a specific position for periods up to 25 seconds. Interestingly, these patterns of maintained attachment often involved suckers from just one side of the arm (see Figs. 3A-B for examples of this as a horizontal stripe pattern).

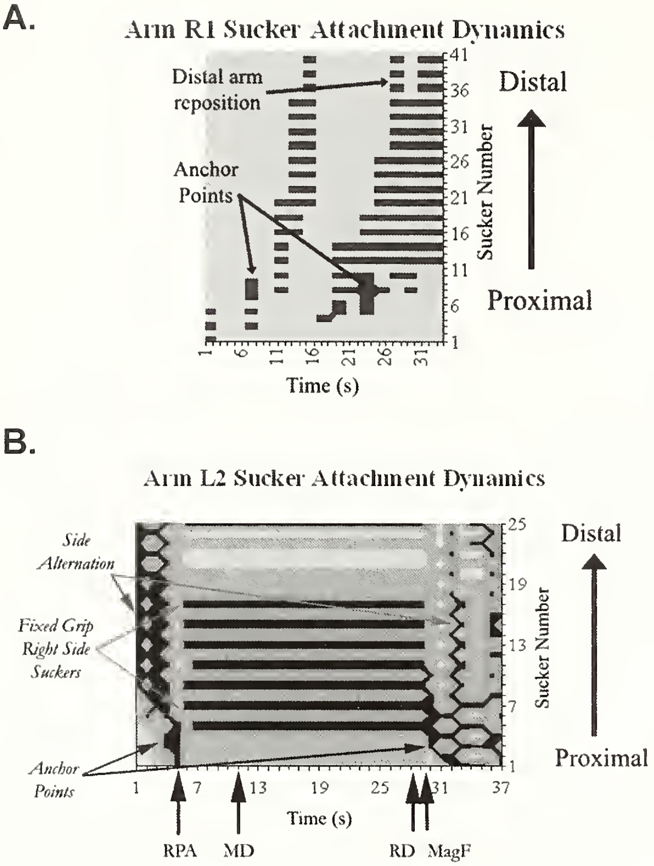

Figure 3. The attachment state of suckers on the arms of two octopuses. The horizontal axis is time measured in seconds. The vertical axis is the sucker number, arranged in sequential order proximally to distally along the arm. The sucker numbers are relative: they are not numbered from the first sucker on the arm. They are simply numbered from the sucker closest the arm base that was visible during the scored observation period. Observations are continuous from that first observable sucker: odd numbered suckers are all on one side of the arm while even numbered suckers are on the other side. Shades of gray represent attachment state. Correlated activity of the arm is marked along the left margin. A, State of 41 suckers on the right first arm (R1) observed over a 37-s period. Dark portions of the plot show attachment, light gray indicates that the sucker was free or in contact with the glass but not sealed. During this period the octopus oriented this arm vertically along the surface of the tank wall as a relatively straight segment. The base of the arm was positioned, and the arm was whipped twice from base to tip in a series of stepped waves. The tip moved freely, its suckers were not scored, and the base was not visible. The horizontal striped pattern results from only suckers on the leading edge of the arm attaching to the surface. Two points marked "anchor points" are groups of attached, adjacent suckers at the start of the move. B, Attachment patterns of 25 adjacent suckers as the animal raised the dome discussed in the text. The grey levels progress from lightest to darkest to indicate "free", "contact", "partially attached", and "attached" states. The arrows marked RPA, MD, RD, and MagF point to times when the animal **R**e**P**ositioned the **A**rm relative to the dome, the animal **M**oved the **D**ome in a sliding motion along the tank floor, the **R**aising of the **D**ome from the floor of the tank first became visible, and when the **Mag**nets holding the dome came **F**ree and no longer exerted a force to resist the octopus's raising. The zigzag patterns visible at the start and end of the plot represent alternate stepping of suckers on opposite sides of the arm. The horizontal striping marks a period of about 20 seconds during which the suckers on just one side of the arm were attached to the dome and during which the octopus was presumably applying a raising force to the dome. Anchor points toward the base of the arm are again visible in this figure. Note, from the absence of light grey, that the majority of the suckers not attached to the dome were in contact with it during this period.

While both the "arm walk" and continuous attachments described above suggest that suckers that were near neighbors often were not simultaneously attached, we also observed occasions when they were. "Anchor points" (Figs. 3A-B) contrast with the large-scale patterns of coordinated activity described above in that they involved 3-5 adjacent suckers. On these occasions, I observed that the arm was moved by a whip-like motion proceeding from the point of attachment (anchor point) distally. During these arm motions, the distal suckers were unattached and the arm was free to move at all more distal points.

Sometimes the animal would move itself along the tank wall or floor by extending an arm, attaching some of its distal suckers, and then shortening the arm to pull the body toward the attached suckers. When this happened, patterns of coordination involving many and then a few suckers were evident (Fig. 4C). A period of widespread attachments involving 12-15 adjacent suckers on alternating sides of the arm preceded the shortening of the arm. This was followed by a local set of attachments of 2-3 adjacent suckers that supplied the fixed point toward which the animal's body was moved.

Intermediate-sized adjacent groups of four to eight simultaneously attached suckers were also observed (Fig. 4B). These were observed proximal to a portion of the arm that was extended out from the plane of the tank surface wall.

Object raising experiments

In initial trials, the animals were simply released into the chamber with the dome. The animals were thus free to approach the dome in any manner and free to use all of their arms. The animals invariably draped themselves over the dome, mouth over the dome apex. In the typical posture, the web was expanded over the dome and the arms fell around the sides and made contact with the tank floor. The move-

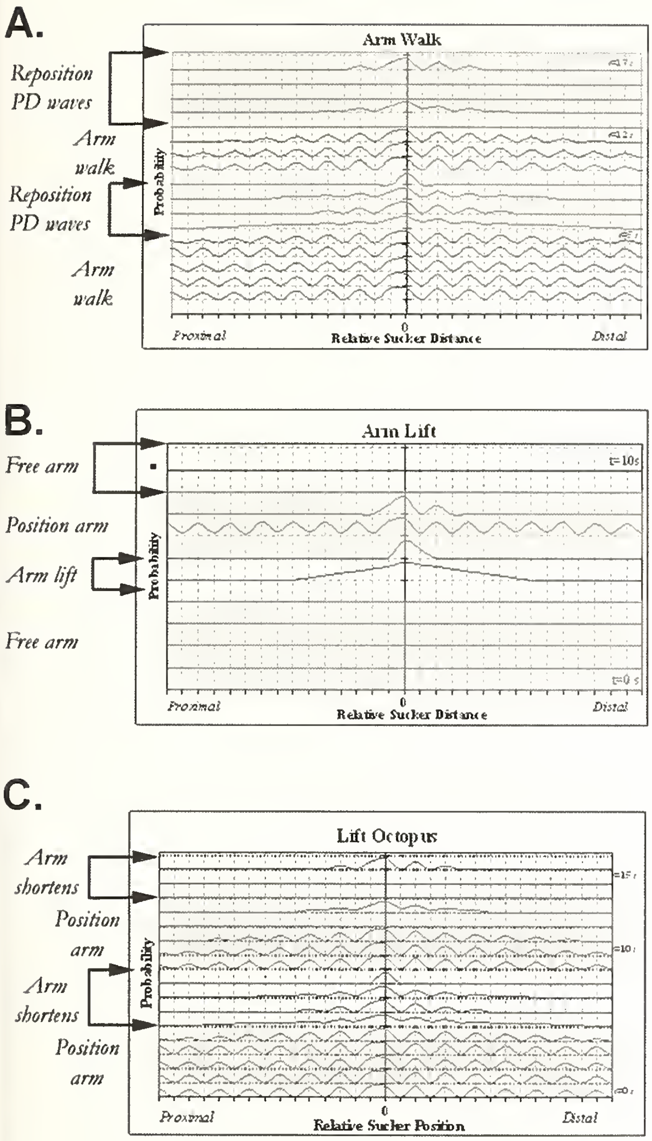

Figure 4. These plots represent the probability of attachment pairs of neighboring suckers during three different types of arm movements. Each line plotted along the vertical axis represents that probability at one second intervals, with earlier traces lower. The horizontal axis represents the distance between neighboring suckers; zero is self (probability always equal to one), negative values are suckers in the proximal direction and positive values are in the distal direction along the arm. Dashed light grey vertical lines mark sucker distance and dashed light grey horizontal lines mark the probability scale (0 to 1) for each trace. A, 41 suckers observed on arm R1 for 20 seconds. During this "arm walk", the octopus moved its arm along the glass with an alternative stepping of the suckers on either side of the arm. This is reflected in the wavy pattern of traces between 0 and 6 seconds and again between 9 and 13 seconds. Between these two periods and after them, the arm was moved by proximal to distal waves along the arm. The widespread patterns of coordinated sucker activity during the walk contrast with local patterns of activity during the wave where anchor points were formed proximally to enable the movement of the arm. B, 10 seconds of observations from 35 suckers on R3. During this period, the octopus moved this arm out away from the surface of the glass using a proximal set of suckers as an anchor for the arm. At 5 seconds, the local neighborhoods spanned 3-5 attached suckers, presumably to support the weight of the arm away from the glass. In the next second, the arm returned to the surface and made several smaller local points of contact. In the following second, a widespread pattern of attachment by suckers on just one side of the arm appeared. This was followed by local groups of suckers in the next second and the movement of the arm from the surface presumably supported by suckers on the other arms. C, Observations of 49 suckers during 16 seconds when the octopus used arm R1 to lift its entire body up the along the tank wall. The octopus projected the arm upward from its body, attached it with distal suckers and then shortened its arm to pull itself upward. The animal repeated this sequence twice during these 16 seconds. In both repetitions there is an initial widespread attachment of suckers on alternative sides of the arms, perhaps probing for a suitable hold, followed by a narrowing to local neighborhoods of attachment during the pulls.

ment and superposition of arms and alteration of suckers made observations of individual suckers and arm usage difficult to assess. However, the general pattern at the moment the dome was raised showed one or more large suckers near the base of the arm(s) attached to the dome, more distal suckers on a variety of arms attached to the floor of the tank with suckers in between unattached to either the floor or the dome. It generally appeared that the lengthening of the arms between the suckers fixed on the dome and those fixed on the tank floor produced the raising of the dome.

Trials with the animal able to reach the dome solely through a hole in a partition permitted unambiguous observation of the actions of the suckers on one arm on the dome and tank floor. In these trials, a pattern of dome-raising similar in some respects to that in the unrestrained animal was observed. Without exception, the arm extended beyond the partition was draped over the dome. There followed a period of adjustment of arm position and repeated attachment, detachment, and reattachment of individual suckers. At the time of dome raising, there were always suckers attached to the dome as well as suckers attached to the floor of the tank, both proximally and distally from those attached to the dome.

On three single-arm trials, the octopus slid the dome a short distance across the floor of the tank before the dome was raised. These slides were distinct from a pull of the arm toward the animal in that they were made with suckers anchored both proximal and distal to the dome as well as on the dome itself. They may have been accidentally produced by forces the animal applied to the dome, but on all three occasions the animal released its suckers on the dome and tank floor and repositioned its arm before continuing its efforts to obtain the crab.

All the animals showed a preference for using suckers in the middle of the arm for this task over those at the base or tip. In all six trials with strong and in all six trials with weak magnetic force, the animal used the suckers on the middle of its arm to attach to the dome and the adjacent tank floor (Fig. 5B). A sign test for six trials indicated that these outcomes were unlikely to be due to chance ($S_6 = 6$, $P < 0.05$).

The number of suckers used in strong and weak-magnet trials differed (Fig. 5A). In trials requiring less force, the animals used a mean of 10.16 ± 1.47 suckers while in trials requiring more force they used 15.67 ± 2.58 suckers. While this difference is in the expected direction, a Student's *t*-test for paired samples did not show that this difference was significant [$t_6 = 1.99$, $P < 0.08$].

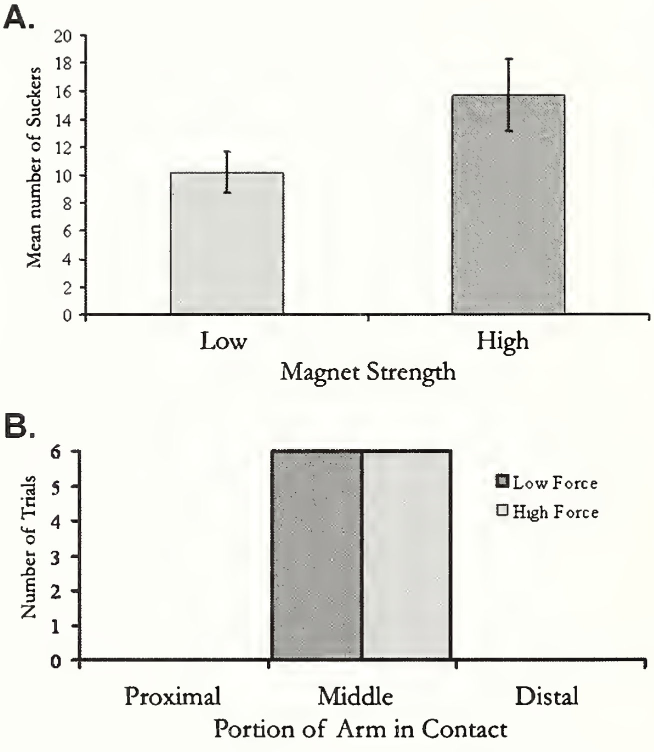

Figure 5. Summary of the results from the dome-raising experiment. A, The average number of suckers used to raise the dome under conditions of high and low force. There is a trend for the number of suckers used to increase with the force required. The means are across animals and the error bars show standard deviation. B, The portion of the arm, divided into rough thirds, used for the raising task during the trial. In all trials all octopuses used the middle portion of their arm regardless of the force required to complete the task.

DISCUSSION

Here I report both local and distant coordination between suckers. Overall, the results of these studies are consistent with the hypothesis of active arm-sucker coordination and inconsistent with the model of exclusive reflexive sucker control. This conclusion is in agreement with observations made in the course of earlier studies of arm control and tactile discrimination in octopuses (Wells and Wells 1957a, Rowell 1963, 1966, Altmann 1971, Wells 1978, Gutfreund *et al.* 2006).

The patterns of sucker use in freely behaving octopuses varied with the behavior in which the octopus was engaged. Octopus, under conditions in which many suckers were in contact with the surface, attached only a subset of suckers—often a very small subset. A reflex-based mechanism, in which suckers attach when stimulated by an available surface, would show much greater proportions of suckers attached on the types of surfaces used in these studies. This suggests differential control across groups of suckers, at least in the form of inhibition or excitation of attachment at selected suckers, based on information about the overall purpose and state of the ongoing behavior of the animal.

The details of the patterns of sucker use during "arm walk", "arm lift", "octopus lift", and other patterns not described in this report suggest even richer forms of information sharing along the arm to determine which suckers will attach and which will remain free at any given moment. To walk the arm perpendicular to its long axis, the suckers on opposite sides of the arm must be differentially attached and detached in opposing phases. Antagonistic pairs of extrinsic muscles within individual suckers need to pull and push with appropriate timing while the sucker is attached to supply the force necessary to move the arm. This and the other observed patterns of inactivation in adjacent sucker pairs demonstrate a side-to-side level of control of sucker attachment within the arm. "Arm walk" would be facilitated with information about the attachment state of each sucker available to the coordination centers, although a strictly feed-forward system can be imagined. The "arm lift" and "octopus lift" examples break the side-to-side coordination patterns of the "arm walk" by allowing adjacent suckers to attach simultaneously and suggest a different type of sucker control. While the arm-walk patterns are widespread, the sucker attachment patterns in "arm lift" and "octopus lift" are local, presumably concentrating strong attachment forces where they are needed to contribute to the ongoing behavior. The fact that one of these occurred near the base of the arm and the other near the tip indicates that this control is distributed along the arm and not localized, in this case, to certain portions.

These observations raise the likelihood of many levels of control and coordination of suckers: locally along the arm in neighborhoods of many scales as well as potentially each of those scales in conjunction with the central nervous system.

This absence of localization or, put more succinctly, coordination of distant suckers, is consistent with the known neuroanatomy of the arm. The basic unit (Fig. 1B) is repeated for every sucker down the length of the arm. Above each sucker the associated brachial ganglia is in a position to share information supplied by its sucker with the adjacent ganglia to support such inter-sucker coordination (Graziadei 1971). In a purely reflexive sucker control system, the nerve running from the brachial ganglia to the sucker ganglion (Fig. 1B) would not be required. Only a local circuit from the sucker ganglia to the sucker muscles and from the sucker receptors to the sucker ganglia (Fig. 1B) would suffice. Taken together, our results suggest that the flow of information between the brachial ganglia and their corresponding suckers is not a one-way sensory channel to inform the arm ganglia and possibly the brain about the state of a given sucker. It is instead a two-way channel in which usable information flows from the adjacent suckers to each sucker ganglion and/or sucker. The question of whether or not individual suckers are sometimes activated without influencing other suckers (*i.e.*, through local connections involving a sensory motor arc from the sucker through the sucker ganglion to the sucker muscles without involving brachial ganglionic connections) remains open.

Specialization of sucker operation, to the extent that it exists, is probably physical in nature, following the proximal to distal taper of the arm. Given that larger suckers are capable of supplying greater adhesion forces (Smith 1991) and that larger suckers are found proximally on the arm (Voight 1993), it follows that tasks requiring greater attachment force will likely call for the use of the suckers toward the arm base. Alternatively, they might require the use of more than one sucker since their adhesive force is additive (Smith 1991). In the dome-raising experiment, both of these responses were observed under conditions that varied the required force. The results showed that as the force required to raise the dome increased, so did the number of suckers used. While this result only approached a traditional significance level of 0.05, the trend was in the direction to support our hypothesis. It is worth noting that variability in force generated to hold the dome down was due to the placement of the electromagnets and that the change in required force was only 28% above the force required to raise the dome alone. It is likely that improvements of this method would reduce experimental error and increase the statistical power of the experimental design. The use of the portion of the middle third of the arm in all trials was contrary to my *a priori* expectations. The larger suckers of the proximal third of the arm were able to reach the dome and I had expected the animal to employ the larger suckers preferentially. The result suggests that, perhaps, the greater flexibility of the middle portion of the arm offered an advantage in completing this task: rather than forming single strong point of attachment on the dome and pulling, the animal produced attachments on the dome and the tank floor on both sides. Thus a trade-off between attachment force and positioning flexibility may occur. The period of probing, contact, and varying attachments/detachments and reattachments that preceded the raising of the dome is consistent with this idea. Information about the force required to raise the dome coming from the suckers could inform arm repositioning. Together these results lead me to tentatively conclude that information about sucker state is available to the arm-control circuits to inform the guidance of arm movements. Given the limitations mentioned above, this conclusion requires confirmation with a more powerful experimental design.

A recent study (Byrne *et al.* 2006) reported that freely moving individuals of *Octopus vulgaris* Cuvier, 1797 that were engaged in visually-guided reaching tasks preferentially make first contact with a target object using the middle of an arm. Byrne *et al.* (2006) were concerned with issues of laterality and arm choice and did not report in detail about the use of suckers in the tasks. Their results implicate visual guidance and, therefore, information from the central nervous system, influencing the part of the arm that is applied to the task and are consistent with the results reported here. It is likely that vision also contributed to octopus performance in the dome raising task. The coincidence of the preference for the middle of the arm in different tasks and in different species is interesting and offers a indication for future studies investigating the relative importance of local and central control mechanisms in the octopus.

In summary, the results of these studies demonstrate that arm-sucker coordination exists almost certainly in the form of descending information influencing sucker activity and very likely in the reverse direction, with sucker state influencing arm movement.

As Wells (1978) wrote over 30 years ago, motor problems in octopuses are rarely studied because of the difficulty of working with such flexible systems. Today we say that these systems are "hyper-redundant"—offering many routes of achieving the same end—but we mean the same thing (Walker *et al.* 2006). An octopus arm with 40 suckers (the number of contiguous suckers typically observable in these studies) is a subset of the real arm which typically has about 300 suckers (Voight 1993). Even if we limit the actions of the octopus arm to (1) suckers that can only be attached or free and (2) the sections of the arm that link each pair of suckers to 1 pitch, 1 yaw, and 1 roll, we still find that such an arm can be in $\sim 1.2 \times 10^{24}$ states, an enormous number of degrees of freedom. Such an arm has the potential to interact effectively with surfaces far more complex in shape and texture than the smooth glass surfaces used in these studies. Indeed, and the real octopus arm evolved to work on more complex surfaces (*i.e.*, extracting prey from crevices and burrows, opening bivalve shells, and arranging middens in front of den entrances). The studies reported here scratch the surface of an enormous, unexplored domain of complex control methods that we might understand if Wells' challenge of flexibility were pursued. The octopus proves it is possible.

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Short-term pain for long-term gain: A hypothetical role for the mantle in coleoid cephalopod circulation*

Alison J. King[1] and Shelley A. Adamo[2]

[1] Department of Biology, Life Sciences Centre, Dalhousie University, 1355 Oxford Street, Halifax, Nova Scotia B3H 4J1, Canada, ajaneking@gmail.com

[2] Department of Psychology, Life Sciences Centre, Dalhousie University, 1355 Oxford Street, Halifax, Nova Scotia B3H 4J1, Canada, sadamo@dal.ca

Abstract: Mantle cavity pressures are frequently hypothesized to drive venous return in the high-output circulatory systems of coleoid cephalopods. However, studies using non-invasive, imaging ultrasound on resting cuttlefish (*Sepia officinalis* Linnaeus, 1758) conclude that mantle cavity pressures do not drive venous return. Interestingly, data from cuttlefish showing sustained mantle hyperinflation indicate instead that forces within the mantle's tissues could aid circulation. We hypothesize that alternating contractions of the radial and circular mantle muscles create a bellows-like effect on mantle capillaries. This effect could be propulsive during normal ventilation and jetting but could stop circulation when the cuttlefish is engaged in sustained mantle hyperinflation. Sustained mantle hyperinflation accompanies some behaviors, for example the Deimatic Display. The metabolic consequences of strangulated circulation might limit the duration of these behaviors.

Key words: cardiovascular dynamics, peripheral circulation, mantle cavity pressure, *Sepia officinalis*, veins

The circulatory system of coleoid cephalopods is closed and has two separate loops: one though the gills, powered by the two branchial hearts, and one through the body, powered by the single systemic heart (Tompsett 1939). During exercise, increases in systemic heart rate and stroke volume, combined with increasing arterial pressure (Wells and Smith 1987), result in the work and power output of systemic heart tissue rivaling or exceeding those of mammals (Shadwick *et al.* 1990, O'Dor and Webber 1991). The coleoid hearts are generally considered insufficient to generate such power output and many authors ascribe an accessory circulatory function to the contractions of the coleoid mantle. The mantle encloses most organs (including the hearts, large veins, and large arteries) in a space called the mantle cavity (Tompsett 1939). At rest, the mantle expands and contracts to move water through the mantle cavity and over the gills. Maximum mantle cavity pressures in resting cuttlefish are around 0.16 kPa (King, pers. obs.). During jetting, the muscular mantle contracts forcefully, increasing maximum mantle cavity pressures by over an order of magnitude to at least 5.5 kPa in cuttlefish (O'Dor and Webber 1991), 8 kPa in octopods (Wells *et al.* 1987), and 6.6 kPa in squid (O'Dor and Webber 1991). Could the forces generated by the muscular mantle help circulate the large amounts of blood needed during exercise?

One model suggests that the hearts should contract at the same time as the mantle. The resulting increase in mantle cavity pressure could augment arterial pressure generated by the heart, driving blood to the low-pressure periphery outside the mantle cavity. Additionally, the slightly negative mantle cavity pressures created during mantle expansion could help to pull venous blood back into the mantle cavity from the head and arms and toward the hearts. However, a 1:1 ratio of heart to mantle contractions is not usually observed in octopods (Wells 1978, Smith 1982), squid (Shadwick *et al.* 1990), or cuttlefish (Chichery 1980, King *et al.* 2005, King and Adamo 2006), even during jetting. Moreover, the ratio of contractions between the heart and the mantle can change over time within the same octopus (Johansen and Martin 1962, Wells 1978) or cuttlefish (King *et al.* 2005). It would seem that heart contractions are not tied to mantle contractions in any fixed way.

In a different model, mantle cavity pressures could drive blood flow in the veins, instead of by helping the heart. Pressures have been measured in the vena cava cephalica (probably the lateral vena cava of King *et al.* 2005) and efferent gill vessel of the octopus *Enteroctopus dofleini* (Wülker, 1910) (Johansen and Martin 1962) and the squid *Loligo pealeii* (Lesueur, 1821) (Bourne 1982). In these vessels, there are two overlaid pressure pulses: one that is relatively slow and large and one that is relatively fast and small (Fig. 1A). The slow, large pulse (Fig. 1B) is due to ventilatory movements (Johansen and Martin 1962). The fast, small pulse (Fig. 1C) is due to venous contraction (King *et al.* 2005). Probably due to the large size of the ventilatory pulse, many have suggested that the mantle cavity pressure flattens large, thin-walled veins such as the venae cavae and the efferent branchial vessels. However, for the veins to flatten, the compression-resistant blood inside them would have to move into the adjacent vasculature. To accomplish this, mantle contractions would have to generate pressure differences between the anterior and lateral venae cavae or between the efferent branchial vessel and the systemic heart. Theoretically, the pressures created in the mantle cavity, while large at times, are applied equally to all veins within the cavity, and therefore would not create the pressure differences required to move blood between vessels. So, while the contractions of the mantle create absolute pressure changes in the vessels, they are unlikely to be propulsive because they are unlikely to change the relative pressure between the vessels.

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Empirical data do not support the hypothesis that mantle cavity pressure compresses the veins. If mantle cavity pressures did compress the veins, we would have two expectations: (1) the veins would contract at the same rate as the mantle and (2) the veins would collapse as a unit along their length as mantle pressure increased. Neither of these occurs in experimental observations using imaging ultrasound. First, the lateral venae cavae and efferent branchial vessels do not contract at the same rate as the mantle and, therefore, could not be compressed by it (King *et al.* 2005). Second, the only large vein that does contract at the same rate as the mantle in resting cuttlefish, namely the anterior vena cava, contracts peristaltically and not as a unit along its length (King *et al.* 2005). Furthermore, this vein's contractions become unsynchronized with mantle contractions in a mating female; the vein's contractions remain steady and slow during the rapid and vigorous mantle contractions that accompany the placement of the male's arm in her mantle (King 2005). The anterior vena cava is evidently able to contract independently of the pressures generated in the mantle cavity. It would seem that pressures in the mantle cavity do not compress the large veins.

Not only are contractions of mantle not propulsive in the veins but also they could impede venous blood flow from the head and arm veins into the anterior vena cava. Increased mantle cavity pressure would increase the pressure in the anterior vena cava without increasing pressure in the venous spaces of the head and arms, thus inhibiting the forward flow of blood (Wells *et al.* 1987).

We have found no experimental evidence that supports a role for increasing mantle cavity pressure in venous return. However, some experimental evidence suggests that mantle tissue may play a role in circulation. In this paper, we synthesize this evidence and present a new hypothetical role for

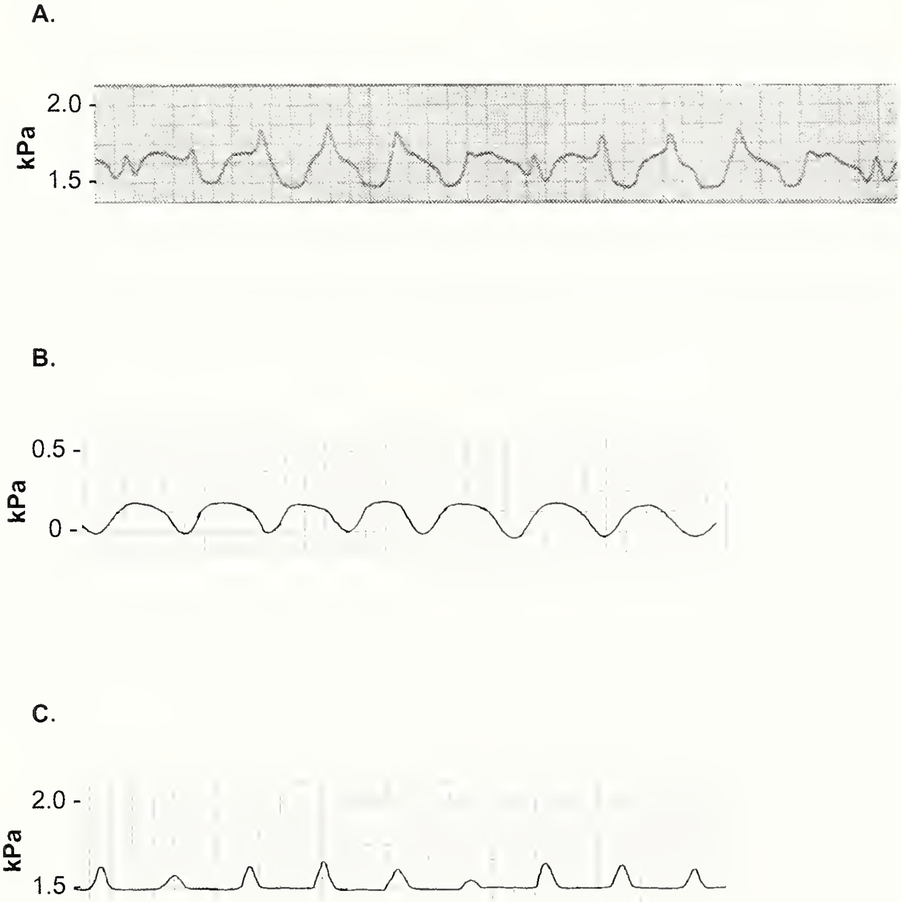

Figure 1. A, Pressure trace from the efferent branchial vessel measured *in vivo* by Johansen and Martin (1962). It consists of two superimposed pressure pulses of different frequencies: a slow and large pulse caused by ventilatory movements (B) and a faster and smaller pulse caused by venous contractions (C). The base pressure of 1.5 kPa is arbitrarily assigned to the pressure trace in C.

the mantle in circulation. We also present additional indirect experimental evidence that supports our hypothesis.

HYPOTHETICAL ROLE FOR MANTLE TISSUE

The mantle may aid circulation by creating pressure within its own tissues, instead of by creating pressure in the mantle cavity. This hypothesis was spurred by experimental results from cuttlefish (*Sepia officinalis* Linnaeus, 1758). When cuttlefish are exposed to a sudden visual stimulus, heart rates drop and mantles hyperinflate for several seconds (King and Adamo 2006). Mantle hyperinflation is an expansion greater than during normal ventilation (King and Adamo 2006). Interestingly, a decrease in heart rate occurred almost simultaneously and proportionally to the mantle's hyperinflation (King and Adamo 2006). This coincidence initiated our interest in the connection between mantle tissue and circulation. To explain the connection we draw, we first present a summary of mantle tissue structure.

Cuttlefish mantle tissue is composed almost exclusively of two muscle types, radial and circular muscles (Fig. 2, Bone *et al.* 1994). Radial muscles contract to expand the mantle cavity during ventilation (Bone *et al.* 1994). Circular muscle contraction constricts the mantle cavity only during heavy ventilation and jetting (Bone *et al.* 1994). Both sets of muscles are partially antagonized by variously arranged collagen tunics and by each other during all but resting ventilation (Bone *et al.* 1994). The collagen tunics keep the decapod mantle the same length so that contractions of the different muscles translate only into expansion and constriction of the mantle.

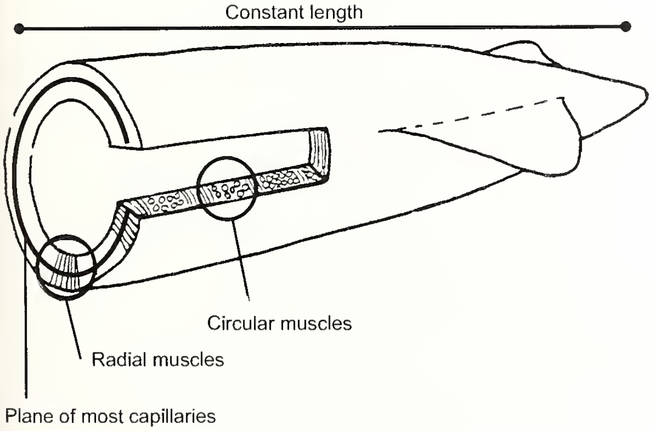

Figure 2. The bands of radial and circular muscles in the decapod mantle and the plane along which most capillaries are aligned. Radial muscles contract to thin mantle tissue and expand the mantle cavity. Circular muscles contract to thicken mantle tissue and constrict the mantle cavity. Collagen tunics ensure that the mantle does not change length during mantle contraction and expansion. Most capillaries are perpendicular to the radial muscles and parallel to the circular muscles. After Shadwick (1994).

Most capillaries in the mantle are oriented perpendicularly to the radial muscles (Fig. 2, Bone *et al.* 1981). We suggest that they could be compressed by radial muscle contraction (Fig. 3A). Conversely, the capillaries are oriented parallel to the circular muscles (Bone *et al.* 1981), and therefore, we suggest, would not be compressed by circular muscle contraction, and in fact could be expanded by it (Fig. 3C). The radial muscles are always used to expand the mantle, albeit minimally, during resting ventilation (Bone *et al.* 1994). In our model, the radial muscles always alternate between creating a gentle force pushing blood out of the capillaries (radial muscle contraction, Fig. 3A) and creating a vacuum in the capillaries that draws blood in (radial muscle relaxation, Fig. 3B). Mantle expansion could thus drive the flow of blood from the mantle into the veins, while mantle constriction could aid the flow of blood from the arteries into the mantle. Even during rest, this would help power peripheral circulation. We hypothesize that this effect would be magnified during jetting when radial muscles contract more vigorously, expelling more blood, and when the circular muscles become active, possibly contributing to capillary dilation (Fig. 3C). The alternating contractions of the radial and circular muscles could create greater peripheral pumping forces to help power circulation during exercise.

More experiments are needed to test this hypothesis. For example, it is unclear whether changes in capillary length would offset changes in their diameter. Certainly, if capillaries were completely occluded by radial muscle contraction, no change in length would compensate. For anything other than complete occlusion, the situation is less clear. Unfortunately, almost no direct observations have been made on blood flow in the periphery which, considering the strength and size of the mantle, hinders our understanding of integrated cardiovascular dynamics (Bourne 1984). Nevertheless, several indirect lines of data support our hypothesis that mantle muscle contraction influences peripheral circulation.

After exposure to a sudden visual stimulus, cuttlefish and octopods hyperinflate their mantles for several seconds, and their hearts slow or stop beating (King and Adamo 2006). Mantle hyperinflation is achieved by forceful contraction of the radial muscles (Bone *et al.* 1994). If our hypothesis were correct, we would expect mantle hyperinflation to dramatically increase peripheral resistance, causing aortic pressure to remain elevated despite slowing or stopping of the heart. Elevated aortic pressure is in fact maintained in

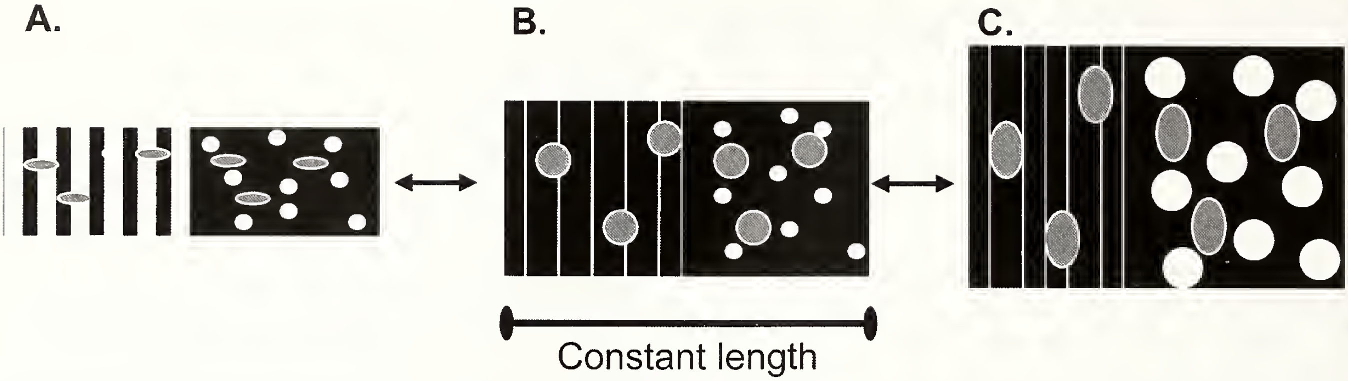

Figure 3. A hypothetical cross-section of mantle tissue during radial muscle contraction (A), relaxation of both muscle sets (B), and circular muscle contraction (C). This cross-section is along the same plane that shows the circular muscles in Fig. 2. The mantle cavity is above the blocks, the open water below. The vertical white lines represent the radial muscles and the white circles represent the circular muscles. Active muscles are represented by heavier lines and circles. The shaded circles represent the capillaries. Capillaries are hypothetically occluded during radial muscle contraction (A) and hypothetically expanded during circular muscle contraction (C). This could propel blood during normal ventilation and jetting.

Enteroctopus dofleini during mantle hyperinflation and cardiac arrest (Johansen and Martin 1962). Furthermore, if our hypothesis were true, we would expect that the veins would fill during cardiac slowing and stopping and mantle hyperinflation, the blood originating from the compressed capillaries in the mantle. The veins and systemic heart in fact do fill at this time (King 2005).

Further evidence is available from jetting cephalopods. Brief mantle hyperinflation starts every jetting cycle. The heartbeat of octopods is interrupted during jetting, although it was not noted whether this is during mantle hyperinflation or water expulsion (Johansen and Martin 1962, Wells *et al.* 1987). During hyperinflation, the radial muscles might compress the mantle capillaries, greatly reducing blood flow through the mantle. The cuttlefish or octopus heart slows or stops at this time, perhaps to avoid dangerous pressure increases in the head and viscera, where blood can still flow. By contrast, when *Octopus vulgaris* Cuvier, 1797 moves using its arms, cardiac interruption is not seen and in fact heart rate increases (Wells *et al.* 1987). It seems that movement does not affect cardiac function unless the movement is achieved using mantle hyperinflation. Interestingly, the mean resistance of the peripheral vessels remains constant or even decreases during mantle contractions (exercise) in octopods (Wells *et al.* 1987) and squid (Shadwick *et al.* 1990). The contractions of the circular muscles during exercise might dilate mantle capillaries, resulting in periods of lowered resistance between the periods of increased resistance associated with radial muscle contraction. The alternating high and low resistance could result in no change or a drop in the mean resistance. Our hypothesis is, thus, consistent with most existing indirect evidence in the literature.

CONCLUSIONS

Currently, we do not have enough data to understand the effects of mantle contraction on the circulation of coleoid cephalopods. The complete picture should integrate the effects of fluctuating pressures in the arteries and veins relative to the periphery during mantle contractions, the effects of the contractions of circular and radial mantle muscles on mantle capillaries, and the effects of the contracting veins. Differences in lifestyle and anatomy may mean this integrated picture differs from one group of coleoid cephalopods to the next. Also to be integrated into the complete circulatory picture are the effects of the accessory vasoconstricting organs found in both cuttlefish and squid on the inside surface of the mantle, around the posterior pallial arteries and veins (Alexandrowicz 1962). Their structure has been well described, but their function is not clear, including whether they contract during contractions of the circular (mantle constriction) or of the radial (mantle expansion) mantle muscles, why they appear on the posterior but not the anterior pallial vessels, and why they do not appear in octopods at all. What is clear is that further research is needed on these accessory vasoconstricting organs and the other factors affecting peripheral circulation.

With new technology being adopted from medicine, the area of integrated cephalopod cardiovascular dynamics promises to be interesting and rewarding in the future. To spur further research, we present a hypothesis for verification—that the mantle could contribute to circulation during ventilation and jetting by alternately compressing and expanding the capillaries in its own tissues. However, strong, maintained contractions of the radial muscles (sustained hyperinflation) may strangulate blood flow (King and Adamo 2006). What might be useful during normal ventilation and locomotion might, thus, be non-adaptive in some acute cases such as the sustained hyperinflation exhibited after a sudden stimulus. This hypothesis is supported by indirect evidence from the literature but requires further investigation. If our hypothesis were supported by direct evidence, it would have consequences for the behaviors that involve sustained mantle hyperinflation, such as the Deimatic Display. The duration of such behaviors may be limited by how long a given animal can forgo normal circulation.

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A new approach to octopuses' body pattern analysis: A framework for taxonomy and behavioral studies*

Tatiana S. Leite[1] **and Jennifer A. Mather**[2]

[1] Universidade Federal do Rio Grande do Norte, Departamento de Oceanografia e Limnologia, Via Costeira s/n, Bairro de Mãe Luiza, Natal/RN, Brazil CEP 59014-100, leite_ts@yahoo.com.br

[2] University of Lethbridge, Department of Psychology, 4401 University Drive, Lethbridge, Alberta T1K 3M4, Canada, mather@uleth.ca

Abstract: We systematically analyzed octopus body patterns, based on locations of chromatophore nerve projection, using a proposed new species in the *Octopus vulgaris* Cuvier, 1797 complex, *Octopus insularis* Leite and Haimovici, 2008. Although some taxonomic studies have used body patterns as characters to describe octopus species, a systematic analysis would provide detailed descriptions to assist reliable comparisons among species. This approach also links body patterns, behaviors, and underlying physiology of the chromatophore system. Body patterns were characterized by percent occurrence, areas of skin, and number of components in each. To verify the distribution of chromatic components, skin patterns, and colors among areas of the body, we ran a cluster analysis on occurrence of the components. We identified a total of 16 chromatic, 5 texture, 9 skin units, 6 colors, and 9 chronic body patterns. The cluster analysis showed twelve distinct skin areas of the components' distribution (expressive fields). Smaller fields were found in areas with complex patterns, especially around the eyes, while larger ones were found in areas with simple patterns. These findings differentiate between morphological and physiological units of the display system. The strong degree of similarity among photographs also supports previous taxonomic studies that pointed to morphological similarity within this species from the oceanic islands of northeastern Brazil.

Key words: *Octopus insularis*, behavior

The complex and changing appearance of cephalopod molluscs offers a challenge to the biologist, both in description (Packard and Sanders 1969, Hanlon and Messenger 1988) and in linkage of its body display to specific behaviors (Adamo and Hanlon 1996, Hanlon *et al.* 1999a, Mather and Mather 2004, Adamo *et al.* 2006). Many authors (Packard and Sanders 1969, Packard and Hochberg 1977, Hanlon and Hixon 1980, Hanlon and Messenger 1988, Roper and Hochberg 1988, Mather and Mather 1994, Hanlon *et al.* 1999b) have constructed a repertoire of the body pattern behavior either for one species or to discriminate among species. The problem of species identity is particularly difficult in the *Octopus vulgaris* Cuvier, 1797 species complex (Mangold and Hochberg 1991, Mangold 1998, Söller *et al.* 2000, Warnke *et al.* 2004). Morphological and morphometric analyses have suggested the occurrence of *Octopus insularis* Leite and Haimovici, 2008 (Leite *et al.*, 2008), a cryptic species of this complex from the northeast of Brazil (Leite and Haimovici 2006, Leite 2007), and cataloging body patterns may be a useful addition to separate it from other species and to compare conserved characters (Hanlon 1988, Hanlon and Messenger 1996). Such information can be gained from careful analysis of patterns taken from underwater photographs and film. Recent analysis of films of *Sepia officinalis* Linnaeus, 1758 has used Bayesian probability to identify pattern (Crook *et al.* 2002), Independent Component Analysis to delineate the basic components (Anderson *et al.* 2003), and Principal Components Analysis to look for camouflage units (Kelman *et al.* 2007). Multivariate analyses can also be used to assess symmetry of body pattern expression (Langridge 2006) to understand camouflage (Kelman *et al.* 2007) or to aid in species differentiation, as in the present study.

Body pattern is composed of chromatic, textural, and postural components that combine to produce the final appearance of the individual (Hanlon 1988). Body patterns are controlled at several levels, and chromatophores are the most important elements that define chromatic components. The chromatophores, organized on the body surface into groups designated as "morphological" and "physiological" units, are the smallest units (Packard 1974). The morphological unit is a static arrangement of chromatophore density in the skin, such as patches and grooves, while the physiological units are a dynamic event, resulting from neural activation of a particular set of nerves in a specific area (Messenger 2001). These areas are called "motor fields" or "chromatophoric fields" (Packard 1974, Messenger 2001) and are usually irregular with overlapping boundaries (Packard 1974). These chromatophoric fields depend both on the distribution of chromatophores in the skin and organization of their neuromotor control (Messenger 2001).

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Because chromatophores are innervated directly from the brain, it should be possible to map the projection of the chromatophore nerves onto the body surface to describe the larger units that Messenger (2001) calls "chromatomotor fields." Froesch (1973) found 20 areas of projection of chromatophore nerves on the body surface when he made selective lesions in *Octopus vulgaris*, and Bühler *et al.* (1975) divided the mantle into 23 smaller projection areas, based on 40 nerves leaving the stellate ganglion.

We believe that using photos of living octopus to analyze body patterns systematically, based on locations of chromatophore nerve projection on the body surface would make it possible to link body pattern components to areas of nerve projection, as well as linkages to behavior states. Body patterns were characterized in percentage of occurrence, locations and numbers of components, and cluster analyses were used to identify groups of similarities among photographs and among octopus body surfaces.

MATERIALS AND METHODS

Juvenile and adult specimens of *Octopus insularis* from the Fernando de Noronha Islands, a northeastern Brazilian oceanic archipelago (03°51'S, 32°25'W) located 345 km northeast of Cape San Roque, Brazil, were photographed from 1999 to 2005, during walking trips near shore, snorkeling, and scuba diving. They were found at a depth of 0.1 to 25 m, in areas of rock, rubble, and small sand patches, with water temperature ranging from 23 to 27°C. We obtained 365 photographs with a digital Canon Power Shot and Sony S50, both 5.0 megapixel, from 93 octopuses.

Conspicuous characteristics of body patterns, behavior, and habitat were used to exclude photographs of three other species of Octopodidae from the analysis: *Octopus hummelincki* Adam, 1936 was identified by the ocellus below the eyes; *Octopus defilippi* Verany, 1851, found only on sand and mud in the Rocas atoll, was identified by the white-cream color; and *Callistoctopus macropus* Risso, 1826, a nocturnal species, could be easily identified by conspicuous white spots all over the body. All photographs without the characteristics cited above but with characteristics common to *Octopus vulgaris* (Nesis 1987, Voss and Toll 1998) were classified as *Octopus insularis*. A subset of photographs was chosen based on an *a priori* assessment of image quality, definition, portion of the body visible, and body pattern. We chose 65 photographs from 23 animals that showed at least three areas of the body (*e.g.*, mantle, head, and at least one arm), were of high quality, and were not the same pattern, date, and individual. We determined different behaviors, body patterns, and their relationships based on Packard and Sanders (1971), Roper and Hochberg (1988), and Hanlon *et al.* (1999a), plus the components (chromatic, textural, colors) and skin patterns present in each photograph (Tables 1-2). The components and skin pattern were catalogued based on Packard and Sanders (1969), Roper and Hochberg (1988), and Mather and Mather (1994) (Table 2). The colors were the five cited by Messenger (2001) for *O. vulgaris*, plus Blue-Green, derived from the iridophores (Florey 1966, 1969, Messenger 1974, Cooper *et al.* 1990).

Presence of components and colors throughout the body and within each body pattern

We analyzed the photographs for presence or absence of each chromatic and textural component, color, and skin

Table 1. Behavior states and the chronic body patterns identified in photographs of *Octopus insularis* from Fernando de Noronha, Brazil.

	Definition	Acronyms	References
Behavior states	Inside Den	D	
	Outside Den	OUT	
	Hunting	H	
	Swimming	S	
	Mating	M	
Body patterns	Blotch-light blotch and spots spread throughout more than 50% of the skin surface	BL	see chronic Mottle pattern in Hanlon *et al.* (1999)
	Dymantic	D	Packard and Sanders (1971)
	Dorsal Light-Ventral Blue-Green	DL-VBG	see counter-shading pattern in Hanlon *et al.* (1999)
	Mottle	M	see Packard (1969)
	Uniform Dark	UD	see Flush in Roper and Hochberg (1988)
	Flamboyant	F	variation of Packard and Sanders (1971)

Table 2. Components (chromatic, texture, and skin pattern) determined from photographs of *Octopus insularis* from Fernando de Noronha, Brazil (based on Packard and Sanders 1969, 1971, Mather and Mather 1994). R, restricted to specific body area.

Components		Acronym
Chromatic	Alternate bands (light/dark)	ABA
	Alternate light/dark around the eye (R)	ABE
	Brown-yellow blotch	BB
	Blue-green around the eye (R)	BGE
	Black hood	BH
	Dark bar across the eye (R)	DBE
	Dark blotch above eye (R)	DBA
	Dark spots	DS
	Longitudinal dark strip	LDS
	Light blotches	LB
	Purple around suckers (R)	PS
	Red bar across the eye (R)	RBE
	White bar across eye (R)	WBE
	White spots	WS
	White dots	WD
	White V (R)	WV
Textural	Big papillae (>1 cm)	BP
	Small papillae (<1 cm)	SP
	Smooth skin	S
	Rugose skin	R
	Textured skin (skin with a large number of small papillae)	T
Skin pattern	Alternate bands	AB
	Bars	BR
	Blotch	BL
	Dark smooth	DS
	Light smooth	LS
	Reticulate dark	DR
	Reticulate light	LR
	Reticulate mixed (dark and light)	R
	Red/white reticulate	RWR
Colors	Yellow	Y
	Red	R
	Brown	B
	Black	BL
	White	W
	Blue-green	BG

pattern on forty-nine parts of the body (Fig. 1A). These body parts were delineated based on the projection of chromatophore nerves onto the body surface (Froesch 1973, Bühler *et al.* 1975), plus additional divisions in the areas that were much too large, such as mantle and arms (Fig. 1A). Classification of the arms position followed Mather (1998), with the right and left arms numbered: 1st, 2nd, 3rd, and 4th (corresponding to the areas 7, 8, 9, and 10). Within a single arm, the proximal areas were categorized as 1 and 2 and the distal areas, 3 and 4.

To verify occurrence and area of each component and color throughout the body, we calculated: (the number of areas in which a component appeared in the photograph analyzed)/(total areas of body analyzed in the photograph) × 100. For example, the Dark bar in the eye (DBE) occurred in two areas of the body and we analyzed 30 areas in this photograph, so the occurrence for this component would be $(2/30) \times 100 = 6.7\%$ (see Appendix 1 for details). We considered that components with 80% or more occurrences in an area could be considered typical for this area, and with 50-80% could be considered common for it.

To verify the distribution of chromatic components, skin patterns, and colors among the areas of the body, we ran a cluster analysis based on occurrence of all these components, except Brown and White, throughout the areas. These two colors were not considered because they were found throughout the body.

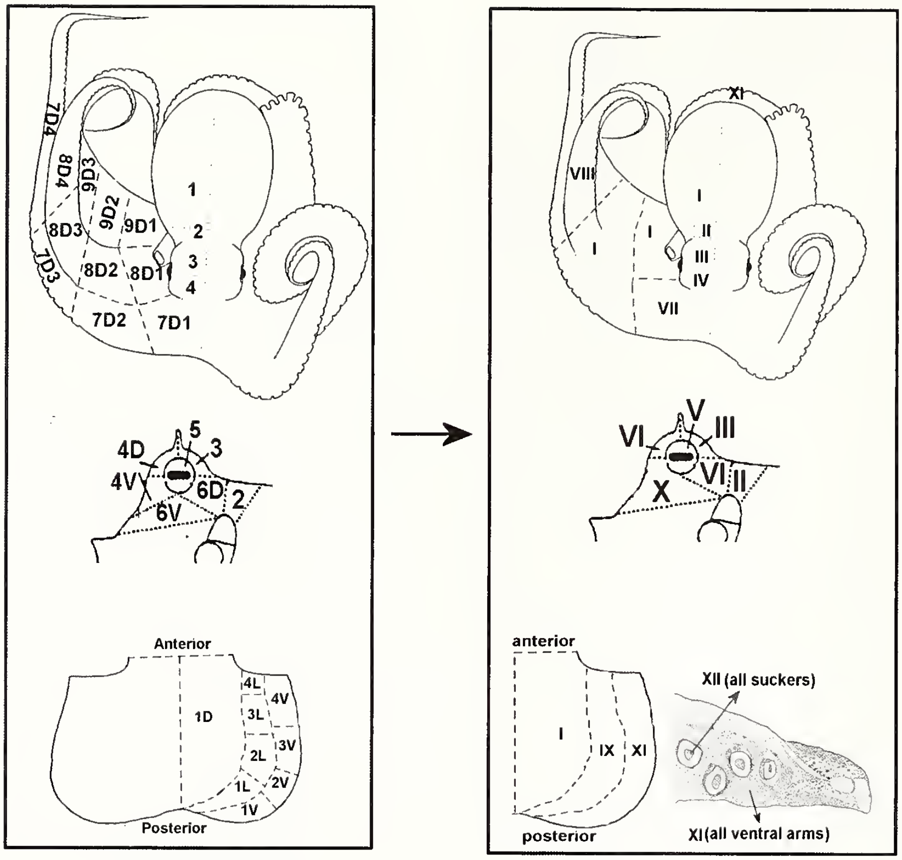
Typical and common components for the body patterns and species

To verify the degree of relationship that each component, skin pattern, and color had with each body pattern, we determined the mean occurrence of each component for the main body patterns: Mottle, Blotch, Uniform Dark, Dymantic, and Dorsal Light-Ventral Blue-Green. We calculated: (the times that each component appeared in the photographs within a distinct body pattern) /(total of photograph analyzed with this body pattern) × 100. For example the DBE appeared in 5 of 10 photographs classified as Uniform Dark, so the occurrence for this component would be 50% in this body pattern. If a component appeared only in areas of the body that were not present in the photograph analyzed, the photograph was not included in the total.

We considered that components with 80% or more occurrences in all body patterns could be considered "typical" for the species and with 50-80% could be considered "common" for this species (Appendix 2). The components with ≥80% or more only in one body pattern were considered typical for them; those with 50-80% were considered common.

Similarity among photographs

To determine how many groups of animals could be differentiated from the photographs, a cluster analysis was run, taking into account the presence and degree of expression of each component throughout the parts of body. A cluster analysis encompassed a number of different classification algorithms to join together objects (photographs and skin areas) in successively larger clusters, using some measure of similarity or distance (Statistic Program Contents 2000). A typical result of this type of clustering was a hier-



A - before cluster analysis

B - after cluster analysis

Figure 1. Areas of the body of *Octopus insularis* onto which skin patterns were projected: A, Areas of protection of chromatophore nerves to the skin (based on Bühler *et al.* 1975, Froesch 1973). B, Areas of common pattern expression, as determined by cluster analysis of occurrence of the pattern components.

archical tree that put together the cases that had similar indices and separated the ones with different indices.

RESULTS

We found sixteen chromatic components, five textures, nine skin units, six colors, and six chronic body patterns in five different behavioral states (Outside den, Inside den, Swimming, Mating, and Hunting) (Tables 1-2). Although many different body patterns were found in each behavioral state, some of them were more common than others. For instance, Mottle (Fig. 2A) was common in Hunting (60.9%) (Table 3).

Presence of components and colors throughout the body and within each body pattern

Seven of the chromatic components were restricted to specific areas of the body: (1) the White V at the proximal part of the arms 1R and 1L (area 7D1) (Fig. 2A); (2) blue green around the eyes (areas 3, 4D, and 6D) (Fig. 2A); (3) alternate bars on the distal parts of the dorsal arms (areas 3 and 4) (Fig. 2A); (4) bar across the eye (areas 4D, 5, and 6D) (Fig. 2B), usually dark but sometimes red or white; (5) alternate light/dark around the eye (areas 3, 4D, and 6D) (Fig. 2B); (6) Dark blotch above the eye (area 2) (Fig. 2C); and (7) Purple around suckers (Fig. 2D). Components described in more than one area around the eye, such as DBE and BGE, could vary their location to one, two, or three of the areas at a given moment. For example, DBE could be present in only 4D, in 4D and 5 together, or in three areas (4D, 5, and 6D) at the same time.

Among textural components, the Small Papillae (Fig. 2F) were spread throughout the body, while Big Papillae (Fig. 2F) occurred disproportionately, but not commonly, on dorsal mantle (1D, 16%) or at proximal-dorsal area of the arms 1R and 1L (7D1, 23%). The skin pattern Light Smooth was typical of ventral areas of the mantle, with 93% occurrence, and Red /White Reticulate on the ventral arms, with >90%.

The colors Brown and White were widespread throughout the body, while all others colors showed some concentration in different parts of the body. Blue-Green was typical in ventral mantle (100%); Red was typical to ventral parts of the arms, the edge of suckers (both with >80%), and common to eyes (>60%). Yellow was common in areas around the eyes (>60%).

Some components could occur in different proportions throughout the body across distinct Body Patterns. For example: Light Blotch (LB) appeared in 40.9% of the body areas in Blotch, while it appeared in just 4.2% of the areas in Mottle. White Spot was the most common component of the areas in Mottle (57.7%), while in Dymantic it appeared just in 9.9% (see Appendix 1).

Looking for clusters among the body areas

Cluster analysis of occurrence of the components throughout the areas showed twelve distinct groups (Fig. 1B, in Roman numerals and Fig. 3), seven composed of single nerve projection areas (2, 3, 4D, 5, 6D, 4D, and suckers) and

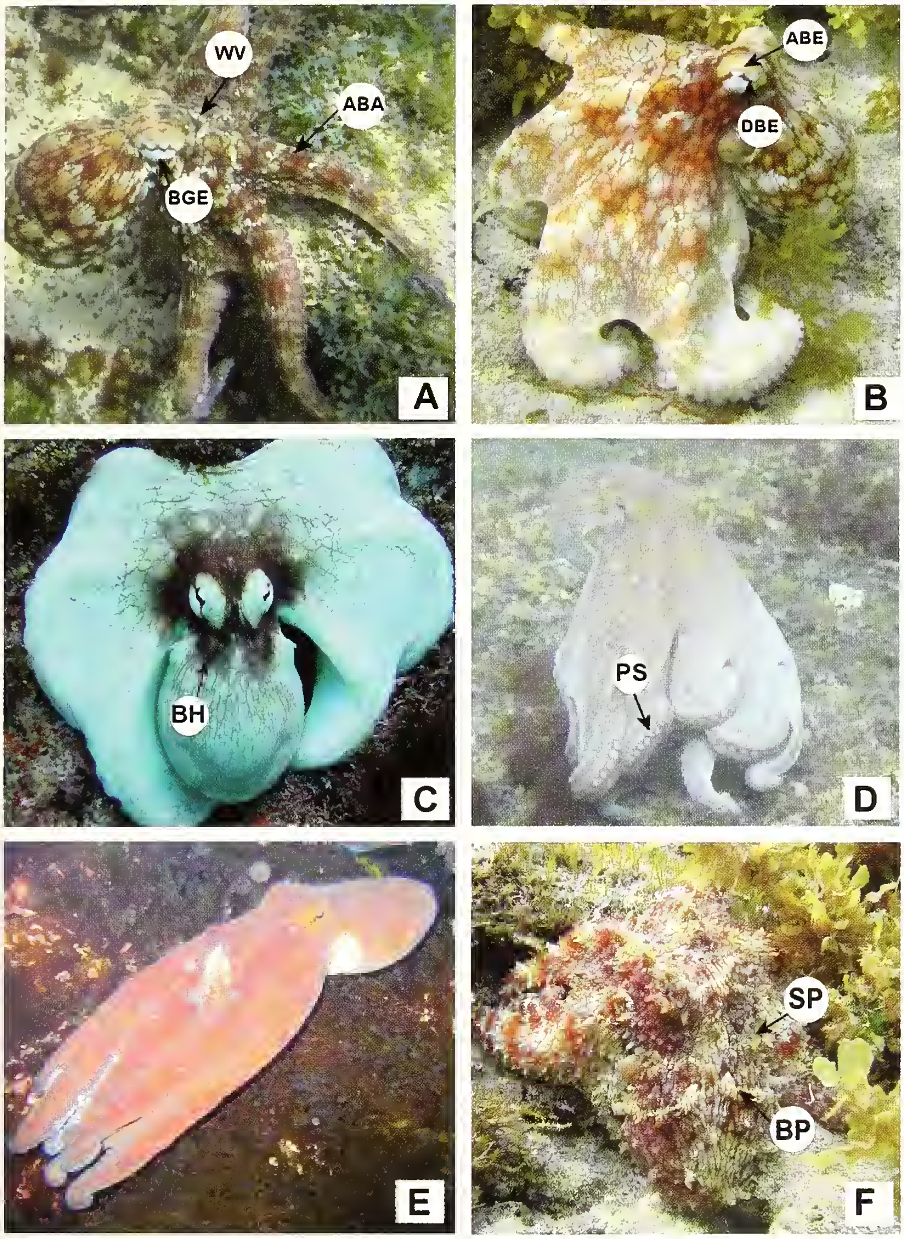


Figure 2. Six body patterns, seven chromatic, and two textural components identified in photographs of *Octopus insularis* from Fernando de Noronha, Brazil: A, Mottle; B, Blotch; C, Dymantic; D, Dorsal Light-Ventral Blue-Green; E, Uniform dark; and F, Flamboyant. WV, white V spot in the middle of the dorsal head; BGE, Blue green around the eyes; ABA, Alternate bars on arm; DBE, Dark bar across the eye; ABE, Alternate bars across the eye; BH, Black hood on mantle; PS, Purple around suckers; BP, Big papillae; and SP, Small papillae.

five of more than one. The analysis showed clustering among all lateral areas of the mantle (1L1, 1L2, 1L3, and 1L4), among two areas of the head (4V and 6V), among distal parts of the 1st, 2nd, and 3rd dorsal arms (7D3, 7D4, 8D3, 8D4, 9D3, and 9D4), proximal parts of 1st, 2nd, and 3rd dorsal arms, including 7D1, plus dorsal mantle 1D (7D2, 8D1, 8D2, 9D1, 9D2, and 1D), and among the ventral mantle and ventral parts of 1st, 2nd, 3rd, and 4th arms, plus the dorsal part of the 4th arms (1V, 7V, 8V, 9V, 10D, and 10V). The areas 10D1 and 10D2 were not considered in the analysis because it was not possible to see them in any photograph. Dorsal areas showed a larger number of components (7-10) than ventral arms and mantle did (4).

Typical components, skin pattern, and colors for the body patterns and species

The analysis of occurrence of the chromatic component, skin pattern, and colors for the five common Body Patterns (Mottle, Blotch, Uniform dark, Dymantic, and Dorsal Light-Ventral Blue-Green) allowed us to show that some components were typical to the species or the body pattern. Typical components of the species were Purple Edge on Suckers (<87.5%), Dark Bar Across the Eye (85%), and Red/White Reticulate on ventral arms (100%) (Appendix 2). Other components considered common to the species were: paired white mantle spots (1D) (61%), frontal white V (7D1), blue green around the eyes (3, 4D, and 6D), and alternate arm bars (all >50%).

Only Blotch and Mottle had typical components (>80%). The typical chromatic components for Blotch were Dark Bar across the Eye (DBE), Light Blotch (LB), Blue-Green around the Eye (BGE), and Purple Suckers (PS); for Mottle, they were DBE, White Spots (WS), White Frontal V (WV), Alternate Bands on Arms (ABA), and Purple Suckers (PS) (Appendix 2 and Fig. 2).

Similarity among photographs

The cluster analysis indicated that the photographs formed one large similar group (Fig. 4). This analysis showed similarity among the pictures, based on occurrence of the components throughout areas of the body, despite

Table 3. Occurrence of the six chronic body patterns at the behavior states identified from photographs of *Octopus insularis* ($N = 65$) taken at Fernando de Noronha.

Behavior states/ body pattern	Outside den	%	Inside den	%	Hunting	%	Swimming	%
DL-VBG	2	15.4	0	0.0	0	0.0	5	45.5
Mottle	3	23.1	4	36.4	14	60.9	0	0.0
Blotch	6	46.2	0	0.0	3	13.0	1	9.1
Dymantic	2	15.4	3	27.3	1	4.3	1	9.1
Uniform dark	0	0.0	4	36.4	4	17.4	4	36.4
Flamboyant	0	0	0	0	1	4.3	0	0

differences among Body Patterns, which probably indicated that the specimens belonged to the same species. The cluster analysis just separated three pictures with conspicuous patterns and proportion of components from the larger group: Flamboyant and two pictures of Uniform Dark during Swimming.

DISCUSSION

Body patterns are a useful taxonomic characteristic for identifying cephalopods in the natural environment (Moynihan 1975, Hanlon 1988, Hanlon and Messenger 1996). This study supports this statement using quantitative analyses as well as qualitative ones to analyze body patterns. Although components had different areas of occurrence and degrees of expression, these parameters were uniform enough that almost all pictures were considered similar by cluster analysis. This strong degree of similarity among the pictures classified as *Octopus insularis* from Fernando de Noronha supports previous taxonomic studies that pointed to morphological similarity in this species (Leite and Haimovici 2006, Leite 2007).

Although qualitative analyses are sometimes not enough to distinguish species or subspecies, they can be used as an indicator. A comparison of body patterns of *Octopus insularis* with ones described for *Octopus vulgaris* from the Mediterranean (Packard and Sanders 1969, 1971) and Bermuda (Mather and Mather 1994) showed that some chromatic and textural components occurred in both species. These are frontal white spots (forming a "V" in *O. insularis* and split for *O. vulgaris* from the Mediterranean) (Fig. 2B), mantle white spots (not described for *O. vulgaris* from Bermuda), arm bars, eye bar, black hood, and long papillae on the mantle and head. Otherwise some components such as the extended hood and transverse stripes (chevron), eye ring, head bar mantle shield, and grainy texture were observed for *O. vulgaris* only from the Mediterranean. Some components described in this study for *O. insularis*, such as blue-green around the eye (Fig. 2A) and alternate light and dark around the eye (Fig. 2B), had not been cited for *O. vulgaris* from either region.

The quantitative results showed that only a small number of components can always be observed across different body patterns. These results make it difficult to do a general Body Pattern description for this species, such as that of *Haplochlaena maculosa* (Hoyle, 1883) (Roper and Hochberg 1988). However, some components were strongly related to specific body patterns, and this close relationship was useful to make a solid characterization of the body patterns that will be useful in future research.

Simple body patterns are found in cephalopods with fewer and larger chromatophores, which could generate fewer components, and complex body patterns are found in species with many and small chromatophores, which could generate more components (Messenger 2001), but this may vary within species. Simple and complex body patterns may, therefore, depend on the number of components. The complex ones (*e.g.*, with more components) were observed during Hunting and Outside Den (Blotch and Mottle), while the simpler ones (fewer components) were more common during Swimming (Dorsal Light-Ventral Blue-Green and Uniform Dark). As the octopuses were photographed outside their den in habitats of different complexity including coral reef, bed rocks, and rock shores, the high degree of complexity could be explained if some habitats require complex body patterns to match them. That might be true for *Octopus insularis,* but not for all species: Hanlon *et al.* (1999a) found *Octopus cyanea* Gray, 1849 exhibited little background-matching outside its den. The degree of complexity that body patterns show within the same species or even individual in relation to the environment indicates a great sophistication of pattern use (Messenger 2001) that needs to be evaluated in detail for many species.

Different levels of complexity of distribution can also be found throughout different regions of the body in a single species and may determine the components and patterns that each region can display. During studies of *Loligo opalescens* (Berry, 1911) chromatophores, Florey (1966, 1969)

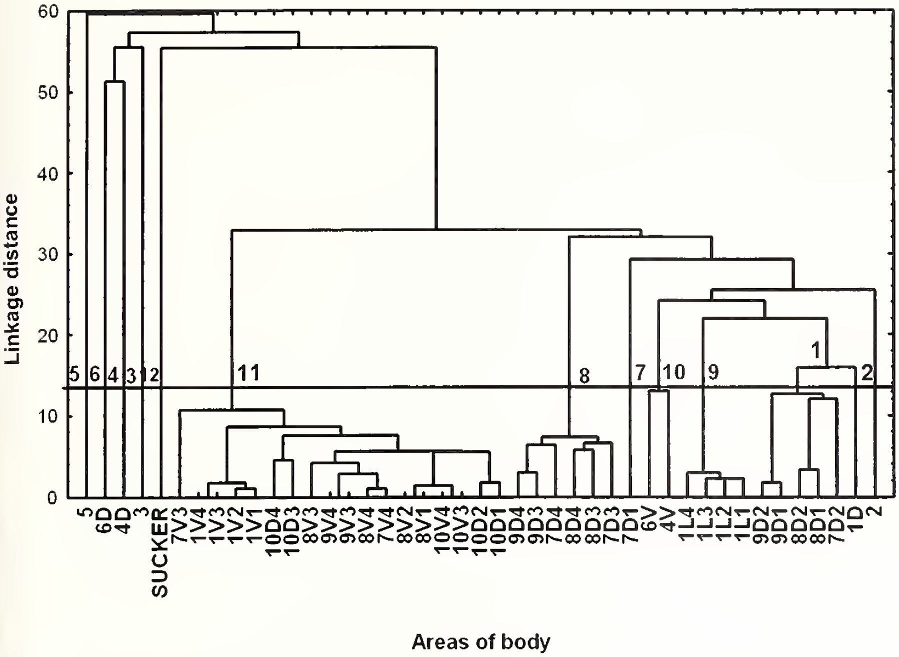


Figure 3. Classification of *Octopus insularis* body skin areas, using cluster analysis. Similarity among body areas was calculated by means of the proportion of occurrence of components, skin patterns, and colors (red, yellow, and blue-green). The line is the cut point, and the numbers indicate twelve different groups of areas on the skin (see Fig. 1B).

found large and sparse chromatophores with single innervation in the ventral mantle, and small and numerous chromatophores with multiple innervations in the dorsal mantle. We also found simple body patterns in ventral areas (mantle and arms) and complex ones in the dorsal areas (mantle, head, and arms). Different degrees of complexity of pattern throughout body areas of an individual can be explained if more complex areas such as the dorsal areas are more visible and vulnerable than others, while less visible areas such as the ventral arms and ventral mantle show simple patterns. Remember, the skin system is widely believed to have evolved as camouflage (Packard 1974).

Differential occurrences of components in different skin areas as defined by Froesch (1973) should have helped us understand the effective projection area of chromatophore fields. When we analyzed the spatial extent of components and patterns, however, we found twelve areas with common patterns that we call "expressive fields" (Fig. 1B). Some of Froesch's (1973) areas, such as the projection of nerve 5 and 3 at and near the eye, do predict the expressive fields that we found. However, not all of his areas match our findings. For instance, the mantle divides into three large expressive fields, not the large number of projections described by Buhler *et al.* (1975). This important finding reflects the division into different morphological and physiological units which is also seen at the lower level (Packard 1974, Messenger 2001). Our knowledge of the projection areas for pattern (Messenger 2001) is improved when the areas used are the expressive fields.

Bringing order to a complex system such as expression of body patterns, analyzing them as located in expressive fields, and linking them to different situations has many uses. The first is the possibility of using them as an additional means of species identification (Hanlon 1988, Roper and Hochberg 1988, Hanlon and Messenger 1996) in conjunction with morphological and molecular analyses. Beyond this, a systematic analysis of the locations of components may shed light on the physiology of the complex, chromatophore-control system (Messenger 2001) and discriminate the many levels of motor fields on the skin. Additionally, linkage of specific patterns to behavioral states has been traced only for a few displays such as those of *Sepia officinalis* (Adamo *et al.* 2006) and *Octopus rubescens* Berry, 1853 (Warren *et al.* 1974) during hunting, and the Passing Cloud of *O. cyanea* to startle potential prey (Packard and Sanders 1969, Mather and Mather 2004). With a more systematic analysis, new linkages of behavior and color pattern may become clear. This kind of analysis can thus be the key to accessing the behavioral plasticity and sophisticated neural control that modern cephalopods have developed (Hanlon and Messenger 1996) through evolution.

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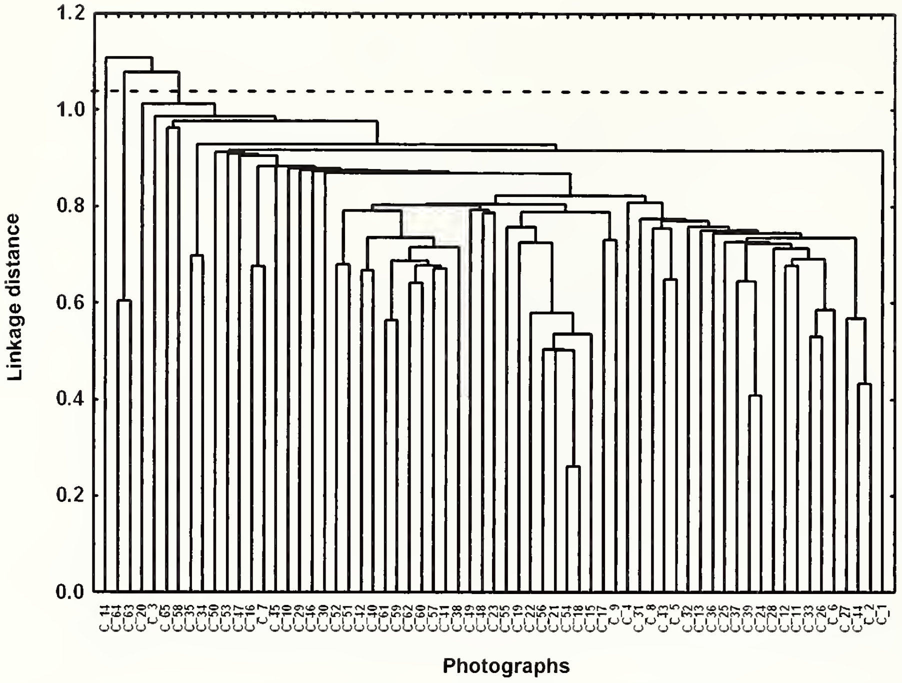

Figure 4. Classification of *Octopus insularis* photographs, using cluster analysis based on the single-linking method. Similarity among photographs was calculated by means of the proportion of the body patterns components through the body (= number of times that components appeared at the areas of the picture/number of areas analyzed).

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Appendix 1. Median percentage of occurrence of the components and colors throughout the body of *Octopus insularis* within the main body patterns: % occurrence = (occurrence of the component or color in the body areas analyzed)/(total parts of body analyzed in the picture) × 100. The numbers in boldface type indicate the commonest components observed throughout the body for each body pattern.

	Components	DL-VBG	Mottle	UD	Blotch	Dymantic
Chromatic components	CR-WBE	1.6%	0.0%	0.0%	0.0%	0.0%
	CR-DBE	9.0%	12.3%	11.4%	12.8%	18.2%
	CR-RBE	0.0%	0.4%	0.0%	0.0%	3.1%
	CR-WS	15.7%	**57.7%**	28.4%	32.1%	9.9%
	CR-WD	4.3%	1.4%	5.4%	3.5%	0.0%
	CR-DS	0.0%	4.9%	5.3%	0.4%	0.0%
	CR-LB	14.8%	4.2%	0.6%	**40.9%**	6.2%
	CR-DBA	0.0%	2.3%	0.4%	2.9%	4.2%
	CR-ABE	1.9%	8.2%	6.9%	2.7%	2.6%
	CR-WV	0.5%	4.1%	3.3%	2.0%	1.3%
	CR-BH	0.0%	0.6%	0.0%	0.0%	18.8%
	CR-ABA	0.0%	19.9%	2.3%	12.2%	0.0%
	CR-PS	2.9%	4.0%	4.3%	3.0%	0.6%
	CR-BB	0.7%	11.1%	2.6%	3.4%	2.0%
	CR-BGE	8.3%	4.7%	8.0%	9.1%	18.6%
	CR-LDS	5.4%	0.0%	0.0%	0.0%	0.0%
Textural component	TE-R	32.9%	47.5%	54.2%	22.1%	29.0%
	TE-SP	9.6%	**55.0%**	38.3%	12.6%	5.2%
	TE-BP	0.0%	13.3%	9.8%	3.0%	0.0%
	TE-S	**63.0%**	36.4%	34.7%	100.1%	62.5%
	TE-T	0.0%	8.9%	7.1%	0.0%	0.0%
Colors	C-Y	22.5%	37.2%	39.7%	22.6%	6.7%
	C-R	4.9%	26.3%	32.4%	16.6%	4.2%
	C-BR	**73.8%**	**87.8%**	**86.8%**	**91.8%**	**75.6%**
	C-W	63.6%	**74.7%**	53.1%	59.6%	52.7%
	C-BG	49.2%	49.7%	20.1%	45.1%	56.0%

Appendix 2. Relationship of components, skin pattern, and colors of *Octopus insularis* with Body Patterns: % occurrence = (the times that component appeared in the pictures with a distinct body pattern)/(total of pictures analyzed with this body pattern) × 100. The numbers at the top of each column indicate the total of pictures analyzed of each body pattern, and the numbers in boldface type indicate the typical components for the *Octopus insularis*.

	Components	Blotch (11)	Mottle (22)	UD (12)	Dymantic (7)	DL-VBG (7)
Chromatic components	CR-WBE	0.00	0.00	0.00	0.00	42.86
	CR-RBE	0.00	4.55	0.00	28.57	0.00
	CR-DBE	**90.00**	**100.00**	**100.00**	**100.00**	**85.71**
	CR-WS	63.64	100.00	83.33	42.86	71.43
	CR-WD	9.09	9.09	33.33	42.86	40.00
	CR-DS	9.09	36.36	25.00	42.86	0.00
	CR-LB	90.91	27.27	8.33	14.29	42.86
	CR-DBA	60.00	62.50	8.33	57.14	0.00
	CR-ABE	36.36	63.64	33.33	14.29	28.57
	CR-WV	36.36	81.82	54.55	0.00	20.00
	CR-BH	0.00	4.55	0.00	71.43	0.00
	CR-ABA	54.55	81.82	16.67	0.00	0.00
	CR-PS	**87.50**	**90.00**	**100.00**	**100.00**	**100.00**
	CR-BB	18.18	50.00	16.67	14.29	14.29
	CR-BGE	90.91	50.00	58.33	100.00	85.71
	CR-LDS	0.00	0.00	0.00	0.00	14.29
Textural component	TE-SP	45.45	95.45	66.67	28.57	42.86
	TE-BP	9.09	50.00	41.67	0.00	0.00
	TE-T	0.00	31.82	16.67	0.00	0.00
Skin pattern	SP-RWR	**No photos**	**100.00**	**100.00**	**100.00**	**100.00**
	SP-AB	54.55	86.36	16.67	28.57	0.00
	SP-DS	0.00	9.09	25.00	14.29	0.00
	SP-LS	90.91	45.45	75.00	100.00	100.00
	SP-DR	63.64	36.36	100.00	85.71	28.57
	SP-LR	27.27	4.55	33.33	85.71	100.00
	SP-BL	90.91	27.27	0.00	14.29	28.57
Colors	C-Y	81.82	81.82	100.00	42.86	85.71
	C-R	72.73	95.45	91.67	42.86	85.71
	C-BR	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
	C-W	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
	C-BG	**90.91**	**100.00**	**91.67**	**100.00**	**100.00**
	C-BL	9.09	36.36	25.00	42.86	0.00

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Observations of deep-sea octopodid behavior from undersea vehicles*

Janet R. Voight

Department of Zoology, The Field Museum, 1400 Lake Shore Dr., Chicago, Illinois 60605-2496, U.S.A., Jvoight@fmnh.org

Abstract: Despite high octopodid diversity in the deep sea, the few opportunities to observe these animals *in situ* limit tests of behavioral predictions made from anatomy. Over the last decade, I have made numerous opportunistic observations of these octopuses using submersibles and remotely operated vehicles. Most commonly seen were octopuses near hydrothermal vents in the North Pacific Ocean at greater than 2200 m depth. Despite the potential submersible-created artifact, the observed behaviors of octopuses of the genera *Benthoctopus* Grimpe, 1921, *Graneledone* Joubin, 1918, and *Vulcanoctopus* González *et al.*, 1998 are reported here. *Benthoctopus* and *Graneledone* differ in wariness and in egg-brooding postures, although both genera produce large eggs from which male hatchlings emerge with clearly developed copulatory arms. In a behavior interpreted as foraging for infauna, octopuses of both genera move the mid-section of their arms through the upper sediment. *Graneledone* seems more common, perhaps because individuals are typically larger and move more slowly. The greater wariness of *Benthoctopus* increases the species' propensity to jet and limits observations and capture. Despite considerable submersible time spent in their habitat, octopuses of *Vulcanoctopus* remain little known; only male specimens are available for study. The few data available indicate that deep-sea octopuses take small prey, as Voss (1988) predicted based on the lack of the esophageal crop and the small posterior salivary glands. If deep-sea octopuses rely on small prey, the need for a crop to serve as a food storage organ is minimized, as is the need for glands that produce venom to subdue large and potentially dangerous prey.

Key words: *Benthoctopus*, *Graneledone*, *Vulcanoctopus*, North Pacific Ocean, embryos

Deep-sea octopodids, defined here as occurring at greater than 2000 m depth, are surprisingly diverse (Voss 1988). Our knowledge of the biology of these animals, however, is limited by the rigors imposed by the deep-sea habitat. The darkness, cold, and high hydrostatic pressure define the habitat and prohibit its direct exploration by air-breathers. In addition to these deep-sea features, the unpredictable distribution of the animals and the expense of conducting research in the deep sea mean that most observations of the animals in nature must be opportunistic. Most type specimens were pulled dead from trawl nets and preserved at sea without being seen by specialists. This contribution summarizes our current, minimal knowledge of deep-sea octopodids and offers a starting point for future research.

Comparative anatomical studies of the Octopodidae reveal apparent convergences among deep-sea benthic octopuses (Voss 1988). The ink sac, a symplesiomorphy of the cephalopods that is thought to be associated with defense against visual predators, is lost. Morphometric studies find that deep-sea and high-latitude octopuses tend to have short arms and small suckers compared to octopuses from shallow water and low latitudes (Voight 1993). Features of the anterior digestive system, including the esophageal crop, radula, and posterior salivary glands, are small or absent in deep-sea octopuses, a pattern that Voss (1988) attributed to their diet of small prey. Although its function was not explicitly stated by Voss, the crop may primarily serve to store food rather than digest it, and large posterior salivary glands produce the venom that immobilizes large, dangerous prey that deep-sea octopuses rarely encounter.

This report summarizes my observations of deep-sea octopuses made over the last decade during research cruises, and images made available by participants in other cruises. The deep-sea octopodid genera *Benthoctopus* Grimpe, 1921, *Graneledone* Joubin, 1918, and *Vulcanoctopus* González *et al.*, 1998 are the subjects of the observations.

MATERIALS AND METHODS

Observations of seafloor animals such as octopuses *in situ* require use of the crewed Deep Submergence Vehicle *Alvin* or Remotely Operated Vehicles (ROVs) such as *ROPOS*, *Jason*, or *Tiburon*. Towed cameras that are lowered from a surface ship to a few tens of meters above the bottom to take images at pre-set intervals also can document benthic octopuses. Regardless of the technique used, undersea assets require bright lights and cameras to detect and record the presence and/or behaviors of any deep-sea animals encountered. The behaviors observed therefore are presumed to be typical, as no observations can be made without the lights and other disturbances the subsea vehicle creates.

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Most dives, and thus most observations, reported here took place in the North Pacific Ocean, in or near hydrothermally active areas of Juan de Fuca and Gorda Ridges, generally at depths of 2000 to 2650 m. A few observations were made near 1550 m depth inside the caldera of Axial Volcano on Juan de Fuca Ridge. Because hydrothermally active areas are of interest to geophysicists, geochemists, biologists, and others, a disproportionate amount of the available submersible and ROV time is spent there. Observations also derive from submersible and ROV dives that targeted off-axis rocky outcrops, such as Baby Bare and Wuzza Bare near 2600 m depth on the Cascadia Basin and Mendocino Ridge at depths of 1550 m. Observations of *Vulcanoctopus* were made on the East Pacific Rise, between 8.5° and 11°N near 2550 m depth.

Observations made during the 17 submersible and ROV cruises in which I participated contribute to this report and are supplemented by photographic and video images provided by other researchers and by submersible crews. Several researchers, notably V. Tunnicliffe and R. Embley, kindly allowed me access to their compiled seafloor images. In addition, some researchers provided octopus image(s), without any comment on the frequency with which the animals were encountered or the total duration of bottom time. Although providing vital information, these sources limit attempts to estimate the frequency with which octopuses were detected, or with which a given behavior was observed. In addition, the density of octopus on the deep-sea floor is not uniform (Voight 2002). In some areas, aggregations of brooding octopuses reached extremely high densities (Voight and Grehan 2000, Drazen *et al.* 2003), yet in "normal" seafloor areas, hours of bottom time may be required before a single octopus is seen.

The observations reported here are by their nature *ad hoc*, as are the collections. Other than brooding octopuses, the longest observations were made of individuals that could not be collected. Conversely, often only minimal observations could be made of collected individuals, as they were collected nearly as soon as they were detected. Octopuses were collected in two ways. Large octopuses were grabbed by the submersible's manipulator arm and placed inside lidded boxes. Smaller octopuses were more often collected in a suction sampler. Octopuses were preserved in buffered formalin in seawater and are catalogued in the collections of The Field Museum, Chicago, Illinois. Catalogue numbers can be accessed through the online database at http://emuweb.fieldmuseum.org/iz/mollusks.php. Mantles of preserved octopuses were opened; if the stomach or esophagus appeared full, the organs were also opened and their contents examined under a dissecting scope.

Characters diagnostic of genera (Voss 1988, González *et al.* 1998) that could be detected without examining the specimen in hand were used to identify the octopuses to genus, with the assumption that the ink sac was absent. Octopuses in the Northeast Pacific with a single row of suckers or with suckers arrayed in a zigzag line, prominent supraocular cirri, and textured dorsal mantle were assigned to *Graneledone* Joubin, 1918 (Fig. 1), the only octopodid genus with a single row of arm suckers known from the North Pacific (Voss 1988). The identity of some specimens with zigzag sucker rows was ambiguous after only brief observation, although with extended observations, the texture of the dorsal mantle and the presence of supraocular cirri were nearly always sufficient to resolve any ambiguity. A mantle fold was seen on some individuals, although this character is not considered to be typical of this genus. Two species of this genus have been described from the east and west North Pacific (Nesis 1982, Voss and Pearcy 1990). Although these species were synonymized by Hochberg (1998) as *Graneledone boreopacifica* Nesis, 1982, because cryptic species may exist, I assigned all individuals to the genus rather than a given species.

Pigmented octopuses with double sucker rows were assigned to *Benthoctopus* Grimpe, 1921 (Figs. 2-3), as the only members of this deep-sea genus have been described from greater than 1000 m depth north of 40°N in the Pacific. The dorsal surface of all members of this genus is thought to be smooth, and none of the available observations indicated otherwise. Among octopuses attributed to *Benthoctopus*, four apparent species could be distinguished. The largest species had long arms and strong reverse counter-shading (Fig. 2); two seemingly moderate-sized, uniformly colored species were distinguished by differences in eye size. The fourth species was *Benthoctopus canthylus* Voss and Pearcy, 1990 which was identifiable by the dramatically enlarged suckers on the dorsal arms (see photos Norman 2000: 210).

White octopuses with a double sucker row (Fig. 4) observed near hydrothermal vents on the East Pacific Rise (EPR) from 8.5°N to 13°N were assigned to *Vulcanoctopus hydrothermalis* González *et al.*, 1998. Their double sucker rows, the unusually translucent white mantle, long thin arms with an uneven surface, and small dark eyes that contrasted strongly with skin color supported the identification. Because the EPR is the type locality for the only known species in the genus, referring these octopuses to that species is conservative.

RESULTS AND DISCUSSION

Seafloor observations of benthic octopuses tend to be unusual, even rare, except in areas where brooding octopuses aggregate (Voight and Grehan 2000, Drazen *et al.*

2003). The large size and similarity of deep-sea octopuses to those of shallow-water, however, mean that deep-sea scientists, regardless of specialty, are often able to recognize the animals. The comparative rarity of octopuses makes observations memorable, facilitating information sharing.

Frequent reports of octopuses near hydrothermal vents, especially those with clams, have been attributed to the availability of clams as prey (*e.g.*, Mottl *et al.* 1998) or of hard substrate as a limited deep-sea resource (Voight 2000a). The occurrence of octopuses near abundant clams that host endosymbiotic sulfide-oxidizing bacteria might suggest that octopuses tolerate exposure to hydrogen sulfide, among the most common and toxic chemicals at chemosynthetic habitats. Clams that host sulfide-oxidizing endosymbiotic bacteria, however, can live in areas with very little sulfide in the water (Fisher 1995). Their extensible foot and blood with a high sulfide affinity allow clams to access sulfide deep in crevices or in subsurface water (Fisher 1995); potential clam predators might be exposed to very low sulfide levels.

Graneledone

Size and density: Most frequently observed in the North Pacific were octopuses of *Graneledone* (Fig. 1) due to their large size and comparatively slow movements. These octopuses, especially at depths greater than 2000 m, were reluctant to jet away from the vehicle. Among 49 preserved specimens, those from comparatively shallow depths (1300 to 2000 m) were typically larger (up to 59 cm total length) with longer arms carrying more suckers (up to 116 per arm) than were those from greater (to 2800 m) depths (up to 48.6 cm total length with up to 86 suckers per arm) (Voight, unpubl. data). Members of this genus were seen on sedimented ocean floor, adjacent to hard substrate, brooding eggs in aggregations on vertical rock surfaces (Voight and Grehan 2000, Drazen *et al.* 2003) but rarely near areas directly impacted by hydrothermal fluid flow. Voight (2000a) reported the global distribution of this genus in reference to areas of chemosynthetic activity.

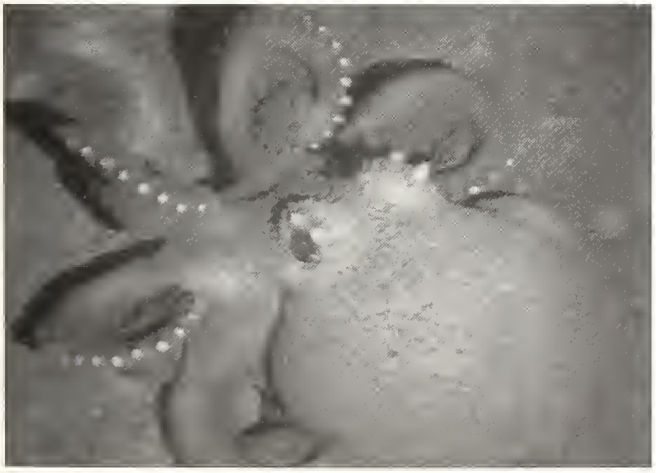

Figure 1. Octopus of *Graneledone* photographed by fixed still camera mounted on front of the submersible *Alvin* during dive 4046 near Wuzza Bare seamount, Cascadia Basin at 2650 m depth.

Behavior: When members of this genus were seen moving across featureless sediment-covered plains, they often used the middle part of each arm as a sediment probe, in a behavior inferred to constitute foraging for infaunal prey. In this behavior, the proximal halves of the dorsal and dorsolateral arms were held nearly straight down to contact the sediment surface. The arm tips extended radially away from the octopus, remaining on top of the sediment, with the middle section under the sediment. As the octopus moved forward, it continually shifted the middle section of its arms through the superficial sediment. Several individuals performed this behavior and some preserved specimens even had a covering of sediment on the distal one-half to two-thirds of their arms. No direct observations of foraging success were possible, as the octopus would have passed any small infaunal organism it collected to the mouth by the suckers on the oral arm surface. Although such movements are very hard to confirm, slight movements of the arms toward the mouth supported the hypothesis that this behavior constituted foraging.

The guts of octopuses of this genus typically contained little. One specimen, from the caldera of Axial Volcano, had eaten snails of *Provanna variabilis* Warén and Bouchet, 1986 and polychaetes characteristic of the hydrothermal vent fauna (Voight 2000b), providing evidence that these octopus feed on small prey, in this case taken from hard substrate impacted by hydrothermal activity. An octopus collected in a non-hydrothermally active area had preyed on a small galatheid crab and a second had an amphipod in its gut.

Octopuses of *Graneledone* were often seen adjacent to hard substrate, where this substrate type was present. With most submersible dives devoted to hydrothermal vents associated with rocks, observations were biased toward hard substrate habitats. Regardless, octopuses appeared to preferentially associate with rare hard substrate in non-vent areas, such as near Ocean Drilling Program (ODP) platforms, long-term instrument deployments, and rocks. The sex and reproductive maturity of the individuals near hard substrates could not be identified.

Color: In situ seafloor observations have limited ability to identify true color, as the proximity, type, and angle of the light source can all affect the perceived color. Octopuses of *Graneledone in situ* appeared to be maroon, orange, purple, or red. Preserved specimens are purple, blue-red, or maroon in color. The skin texture of the dorsal mantle is also quite variable among individuals, from very rugose (Fig. 1) to essentially smooth. The supraocular cirri were always the most readily seen features in terms of skin texture.

Color change was observed in two octopuses of *Graneledone.* In the better-documented observation, the octopus was between the ROV and a second octopus of *Graneledone* when half of its body blanched along the mid-sagittal line. On the other occasion, a lone octopus was approached by the ROV. It blanched, as did the individual reported above, with one side of the body colored normally and the other whitish. The contact between the colors followed the mid-sagittal line.

Egg brooding: Females of *Graneledone* that were seen brooding eggs had attached them to rocks and most often aggregated in areas with rock outcrops. Aggregations are known at 2600 m deep at Baby Bare seamount in Cascadia Basin (Voight and Grehan 2000) and at 1550 m depth on Mendocino Ridge (Drazen *et al.* 2003, Voight and Drazen 2004). Voight (2002) reports that densities of brooding octopuses at Baby Bare exceeded triple those of background areas. Males were present, though rare, among the aggregations. Brooding females invariably were positioned with their dorsal mantle facing outward and the oral surface of their arms touching the rock surface to which their eggs were attached. The total number of eggs produced is unknown, although evidence of at least 50 young was seen in one case at Mendocino Ridge (Voight and Drazen 2004), including eggs and cement from eggs that had apparently already hatched. The female that had been brooding these late-stage eggs showed signs of senescence: slack skin, cloudy eyes, and dull color. Dissection of the collected female revealed conspicuous edema of the tissues, renal organs, and an empty digestive system.

Eggs and embryos: Voight and Drazen (2004) described newly hatched embryos from an egg clutch from Mendocino Ridge that were collected as they hatched. Hatching was likely mediated by the disturbance created by collecting activities, as external yolk sacs were present within the arm crowns. These premature hatchlings were 55 mm long and males could be identified by the hectocotylus and ligula (Voight and Drazen 2004). The single intact egg collected was 39.6 mm long. Earlier, partially developed, 25 mm long ovoid eggs had been collected from an apparently conspecific female at Baby Bare at 2660 m depth (Voight and Grehan 2000). Attempts to model development time of very large cephalopod eggs are not considered to be reliable (Laptikhovsky 1999), but the large size of these eggs and the low temperatures at depth likely mean that their development occurs over a very long period (J. Drazen and B. Robison, pers. comm.). There is no indication that the females feed while brooding.

Benthoctopus

Size and density: Octopuses of *Benthoctopus* were much less commonly seen than were those of *Graneledone.* Whether the few observations of octopuses of this genus compared to those of *Graneledone* are a real difference between the densities of the genera or is, more likely, due to their smaller size, more rapid movements, and/or greater wariness cannot be assessed. The total lengths of specimens of *Benthoctopus* collected during submersible and ROV work were all under 20 cm. Members of the genus *Benthoctopus* were most often seen as single individuals on hydrothermally inactive rocks on the mid-ocean ridge spreading center (Fig. 2). Brooding aggregations were seen at two locations (below).

Behavior: Octopuses of *Benthoctopus* were observed foraging in the sediment as described above for octopuses of *Graneledone.* Individuals of *Benthoctopus*, including *B. canthylus,* were also observed to extend and hold their dorsal arms into the water column on multiple occasions. Octopuses in open, sedimented areas, and on rocks at mid-ocean ridges displayed this posture (Fig. 2). This pose was not seen to result in the acquisition of prey, but small prey may have been manipulated by suckers unobserved. The behavior could also increase chemoreception by the suckers. No octopuses of this genus were observed to change skin color or texture.

Egg brooding: Brooding females of *Benthoctopus* were found at Mendocino Ridge (Drazen *et al.* 2003) and in an

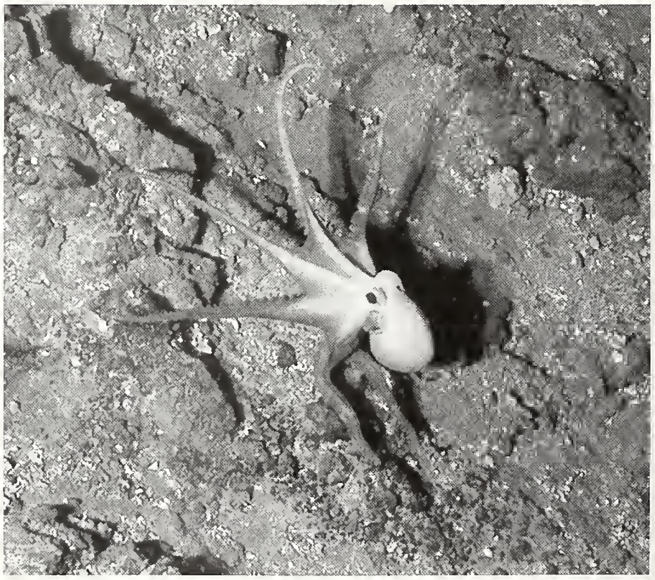

Figure 2. Octopus of *Benthoctopus* photographed by fixed still camera mounted on front of *Alvin* during Dive 4044 near GR-14 or Sea Cliff hydrothermal vent field on Gorda Ridge (North Pacific Ocean) at 2737 m depth.

extremely localized aggregation (Fig. 3) near the GR-14, or Sea Cliff hydrothermal vent field on Gorda Ridge, described by Rona *et al.* (1990). Although located near an area of hydrothermal activity, the octopuses could not be fully demonstrated to be in direct contact with the diffuse hydrothermal fluid in the immediate area. The aggregation was located in 2002 and in 2005 by the ROV *Tiburon* but could not be relocated during an *Alvin* dive in 2004. The aggregations of brooding females of both *Benthoctopus* and *Graneledone* support the hypothesis (Voight 2000a) that substrate availability limits the distribution of brooding females.

A difference between octopuses of *Benthoctopus* and *Graneledone*, based on the estimated 15 females with egg clutches of *Benthoctopus* observed, is that when brooding, these females positioned the oral surface of their arms facing outward and the arm tips in contact with the rock which held their eggs, and potentially with the eggs themselves. Females of *Benthoctopus* often brooded deeper under overhanging rocks than did the larger females of *Graneledone*, possibly due to preference or because their smaller body sizes allowed them to exploit the available niches. However, near GR-14, octopuses of *Benthoctopus* were found closely associated with a nearly vertical wall with their oral, suckered arm surfaces bent backwards to nearly cover the rest of their bodies; in one instance, an egg was visible (Fig. 3). Observations at this site were unfortunately very limited. No collections of late-stage females of this genus have been made.

Embryos: A single embryo of *Benthoctopus* was collected from near GR-14 by the ROV *Tiburon* in 2005. The large (mantle length 7.5 mm; mantle width 7; head width 5.1) embryo (FMNH 309724) had 13.1 mm long arms, each carrying about 66 suckers crowded into two rows. The hectocotylus carried 47 suckers and the ligula was clearly visible on the third right arm tip. The eyes were well developed and chromatophore organs lay just distal to the mantle openings on the lateral body, on the dorsal arm tips, and on the arm base. Few chromatophores, however, were seen on the superficial skin, as those noted on the mantle appear to have been deeper tegumental chromatophores. How near the embryo was to hatching is unclear. The preserved embryo was received with a ruptured egg capsule; the damage was likely inflicted during collection. Although there was no external yolk sac, the torn outer membrane of a yolk sac was present.

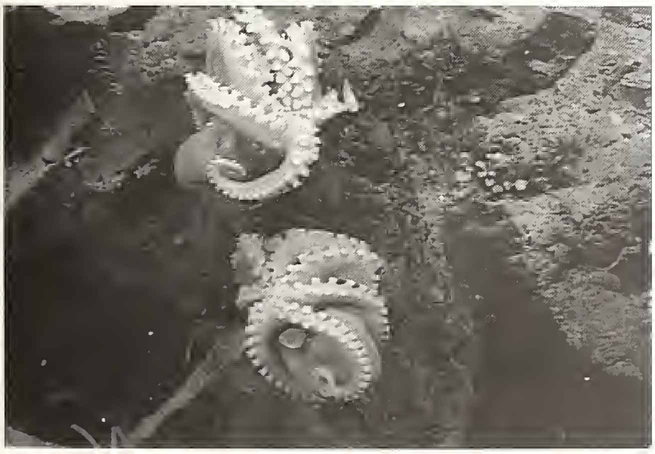

Figure 3. Two octopuses of *Benthoctopus*, one with egg visible, photographed by ROV *Tiburon* during dive T-456 near GR-14 or Sea Cliff hydrothermal field on Gorda Ridge (North Pacific Ocean) at a depth of 2770 m.

Vulcanoctopus

Size and density: Vulcanoctopus hydrothermalis was fairly common at hydrothermal vents on the EPR and at nearby non-hydrothermally-active areas. Typically, and most conspicuously, a solitary octopus was seen moving across bare basalt up to 500 m away from active hydrothermal vents (Fig. 4). Octopuses were also seen in active vent fields, often moving over the surface of giant tube worm (*Riftia pachyptila* Jones, 1981) mounds. More rarely, octopuses of *V. hydrothermalis* were seen at hydrothermal vents in aggregations (Rocha *et al.* 2002, Voight 2005). González *et al.* (2002) reported measurements of 16 individuals of this species; Field Museum specimens are within this size range.

Behavior: Most observations of octopuses of *Vulcanoctopus hydrothermalis* were brief, comprising a solitary individual moving across the basalt substrate in typical octopodid style (Fig. 4). This included random lateral movements of the arms along the surface of the substrate that may

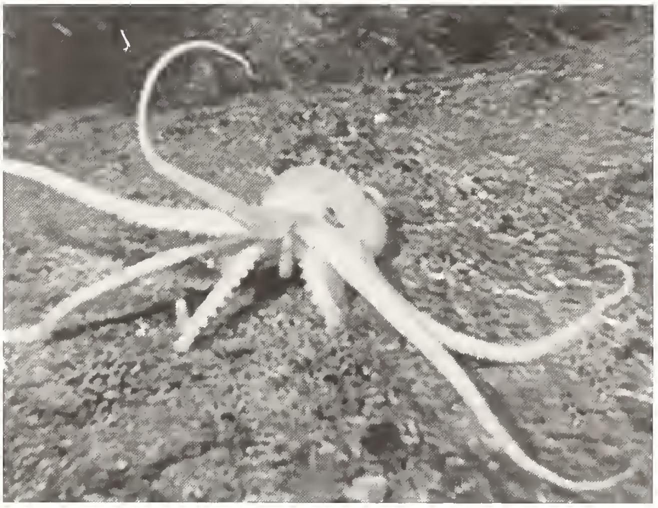

Figure 4. Octopus of *Vulcanoctopus hydrothermalis* photographed on the East Pacific Rise by fixed still camera mounted on front of *Alvin* during Dive 3927, near 9°N at 2495 m depth.

expand the area the octopus searches for potential prey. This species is known only from 27 preserved specimens (the sum of those reported by González *et al.* 2002, and nine Field Museum specimens), all of which are male. This severely biased sex ratio suggests either that the sexes partition habitat or that females are extremely rare. Rocha *et al.* (2002) reported that five apparently male octopuses swarmed a sixth octopus, presumed to be a female, in an attempt to copulate.

Voight (2005) described feeding by a group of these octopuses in which the interbrachial web was used as a cast net to secure members of a dense swarm of the amphipod *Halice hesmonectes* Martin, France, and Van Dover, 1993. In that account, at least 12 octopuses were positioned on four extinct chimneys that nearly ringed a clump of tubeworms. The amphipod swarm, which formed in warm water over the tubeworms, was accessible to the octopuses due to the height advantage the chimneys provided. Such swarms would appear to be an unlikely standard prey for these octopuses, because swarms typically are high enough over the substrate that octopuses would be unlikely to reach them without considerable effort.

Egg brooding and embryos: Females of *Vulcanoctopus* are unknown, as are the egg-brooding habits of the species and its embryos.

CONCLUSIONS

Observations of octopuses of *Graneledone* and *Benthoctopus* apparently foraging for small infaunal animals and of octopuses of *Vulcanoctopus hydrothermalis* foraging on swimming amphipods (Voight 2005) further document that these deep-sea octopuses take small prey. They therefore support Voss' (1988) interpretation of the apparent evolutionary reductions in the anterior digestive system of deep-sea octopuses. Voss (1988) did not explicitly state the crop's function but identified the posterior salivary glands as the source of pharmacologically active venoms used by shallow-water octopus and attributed the reduction of the gland's size to the switch to small prey in the deep sea. Predators ingesting relatively small prey do not require complex venoms, as small prey can be readily subdued. Predators that take prey encountered at low densities have minimal need for food storage, as may be supplied by the esophageal crop. In addition, the radulae of octopuses that feed on bite-sized prey may serve primarily to push individual prey items into the esophagus, rather than tear off bits of venom-soaked flesh, consistent with Voss' (1988) statement that the radula is simplified in deep-sea octopuses. The comparatively small suckers of deep-sea octopuses (Voight 1993) would also function well with small prey (Smith 1996).

Although deep-sea octopuses outwardly are very similar to those of shallow water, behaviorally they have several distinctions. First, although several individuals of *Graneledone* were seen adjacent to hard structures, deep-sea octopuses were never seen to seek or use shelter, other than for egg brooding. Second, when encountered on an open sedimented plain, neither octopuses of *Graneledone* nor *Benthoctopus* made any attempt to camouflage themselves by changing either their color or skin texture. The blanching seen in two octopuses of *Graneledone* may have been a startle reaction, evidence of the presence of the chromatophore system that may be functional in some of these deep-sea octopuses not due to its current adaptive value, but to phylogenetic constraint. Freed of visual predators, deep-sea octopuses apparently rely on immobility or jetting away to evade potential predators.

All octopuses observed and collected have eyes, although those of *Vulcanoctopus hydrothermalis* are reduced (González *et al.* 1998). Because eyes are energetically expensive organs routinely lost by animals living in perpetual darkness, such as caves, their retention by deep-sea octopuses suggests that they are adaptive. One scenario in which eyes would be advantageous is if the octopuses sought bioluminescent prey. If, for example, agitating the sediment during foraging stimulates bioluminescence by infaunal animals, octopuses with eyes could be more likely to capture those prey than would those without eyes. The dark coloration of octopuses of *Graneledone* and the strong reverse ventral counter-shading seen in at least one species of *Benthoctopus* observed here may also be associated with taking bioluminescent prey as the dark ventral color would mask any light emitted from within the web. Although the presence of a light dorsal surface in some species of *Benthoctopus* has been suggested to provide crypsis against a light colored, sedimented background (Voss and Pearcy 1990), ambient light at depths of over 2000 m is negligible.

Most observations reported here were made near hydrothermal vents but cold-seep habitats also support concentrations of biomass that are sustained by chemosynthesis. Octopuses occur in these habitats (Olu *et al.* 1996, Sibuet and Olu 1998, Van Dover *et al.* 2003). Octopuses have also been recently photographed on the West Florida Escarpment seeps at near 3000 m (J. Zekely, pers. comm.), at seeps near 2330 m depth in the Gulf of Mexico (C. R. Fisher and R. S. Carney, pers. comm.). Furthermore, two different species of *Benthoctopus* have been photographed at seeps on Blake Ridge near 2000 m depth (W. Gilhooly, pers. comm.).

Major questions about deep-sea octopuses clearly remain, including life span and reproductive output. Deep-sea octopuses appear to share the life-history strategy common to most cephalopods. Octopus eggs were never found without a brooding female (and were often difficult to see due to her ministrations). All females of both *Graneledone* and *Benthoctopus* observed with late-stage eggs were gaunt, with dull color, slack skin, and cloudy eyes; females are therefore concluded to senesce as their eggs mature. The duration of brooding remains to be determined through long-term observations. The longevity and growth rates of deep-sea octopuses are also unknown. As the logistics of undertaking such research remain difficult in shallow waters, doing so in the deep sea may remain unfeasible. With a robust phylogeny in place, comparative study of the brains of deep-sea octopuses and their color change ability might discover how the elimination of selection for crypsis has affected the nervous system and energy allocation within these deep-sea animals. Until we know the evolutionary history of the octopodids, however, discussions of adaptations or modification to the deep sea remain conjecture.

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To boldly go where no mollusc has gone before: Personality, play, thinking, and consciousness in cephalopods*

Jennifer A. Mather

Department of Psychology, University of Lethbridge, 4401 University Drive, Lethbridge, Alberta T1K 3M4, Canada, mather@uleth.ca

Abstract: The study of molluscan behavior offers intriguing possibilities and promising results, although focused mainly on coleoid cephalopods. Octopuses in particular have enduring individual differences in reactions that are strong enough to be called personalities (Mather and Anderson 1993). Given a floating or manipulable object, octopuses do not always habituate to its presence but may instead perform simple object play (Mather and Anderson 1999). One can argue they have basic concept formation, both in assessment of complex sensory information and choice of motor output. Sutherland's (1963) series of tests on octopus shape discrimination revealed that octopuses had no simple rules but were instead learning what to learn. Anderson and Mather (2007) found that octopuses chose one or more of three methods to penetrate clam shells. Each method used a different effector and prey orientation, all while the clam was under the arm web and thus visual information was unavailable. These different aspects of behavior all indicate cephalopods may have a simple 'primary consciousness' (Mather 2007), integrating perception and learned information with motivation to make decisions about complex actions. Such a conclusion offers new possible directions for the study of molluscs.

Key words: octopus, behavior, cognition

Behavior is often studied in molluscs to understand some other aspect of their functioning, not to evaluate and deconstruct the behavior itself. Behavior is, thus, seen as the consequence of physiology or structure, as in Chase's (2002) book on the behavior of gastropods and its neurophysiological foundation. Alternately, behavior may be evaluated as the outcome of evolution and the one best fit to survival and reproductive success, through foraging strategies (Stephens and Krebs 1986) or sexual selection (Dugatkin 2004). All these approaches are useful, but behavior is a valid field of study on its own, and its study can be based on Tinbergen's (1972) four areas of causation, development, evolution, and function.

Although behavior is emphasized only in the cephalopods—and its complexity revealed—the viewpoint could spread to all molluscs. Behavior is often studied in the coleoid cephalopods because of their learning capacity and high brain-body size ratio (Packard 1972) that is larger than in fish and some birds and approaches that of mammals. They also have excellent visual acuity (Gleadall and Shashar 2004), with eyes convergent to those of higher vertebrates. Cephalopods are well known for variability of behavior, and research (reviewed in Wells 1978) established their excellent learning ability and two storage areas in the brain for visual and chemo-tactile, learned information. Despite criticism of this early research (Boal 1996), the basic findings stand. Hanlon and Messenger (1996) collected information from a wide variety of areas, but focused on body patterns and responses to predators, in reviewing cephalopod behavior.

Advances in all four areas of behavior have given molluscan specialists the opportunity to ask new questions. Development of behavior has been barely touched on, partly because many molluscs, including cephalopods, are small and planktonic at hatching. Several authors (Messenger 1968, Chichery and Chichery 1992a, 1992b, Dickel *et al.* 2000, Darmaillacq *et al.* 2006) have done developmental research on *Sepia officinalis* Linnaeus, 1758 and are reviewed in Mather (2006). Behavioral causation has been studied in learning (Wells 1978), but evolution and function have been studied only in the context of sexual selection in squid (Hall and Hanlon 2001, Jantzen and Havenhand 2003) and foraging strategies of octopuses (Ambrose 1984, Hartwick *et al.* 1978, Mather 1991a, 1991b, Vincent *et al.* 1998). The study of animal behavior is becoming both wider and deeper as researchers learn more, and this should cause malacologists to see the multiple bases of their animals' behavior. West-Eberhard's (2003) masterful combination of evolution, inheritance, environment, and development is one example. Bekoff *et al.*'s (2002) focus on cognition from the animal's own perspective and Baars' (1994) theory of a global workspace as a foundation for simpler consciousness available to non-human species are others. Some abilities that were previously thought of as the sole domain of humans, such as tool use (Beck 1980), play (Burghardt 2004), personality (Gosling 1999), and consciousness (Edelman *et al.* 2005) are being evaluated for non-humans and not just in primates. Such assessments of cephalopods are the subject of this paper.

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Personalities

Individual differences, similar to behavioral syndromes (Sih *et al.* 2004), have recently been rediscovered. The focus on species or group-specific behavior during the second half of the 20th century, both from ethological observation and assessment of learning, was an important and productive advance. Yet in the process, the individual animal was forgotten, and variation among individuals was seen as mostly noise. Although alternative Evolutionary Stable Strategies began to be recognized through game theory (Maynard Smith 1982), this work focused on small groups and not on the individual. However, psychology has a long tradition of looking at individual personalities of humans. Ideas developed in this area, especially theories such as Freudian identity or conflict with one's parents, were not easily transferred to non-human species with quite different experiential bases. Still, Cattell's (1965) factor-analytic approach offered a relatively theory-free evaluation method, using the assumption of individual temperaments. He felt young individuals had a genetic inheritance which combined with environmental pressure to shape personality. Gosling (1999, 2001) has extended this approach to animals, integrated it with research on humans, and this can be applied to cephalopods.

Personality research uses a different experimental paradigm than the usual study for differences among groups as a result of experimental intervention and controls. Instead, we tested 44 individual *Octopus rubescens* Berry, 1953 in three common situations in everyday life: (1) alerting by our opening the aquarium lid, (2) threatening by touching the animal with a test-tube brush, and (3) feeding with a live shore crab (Mather and Anderson 1993). These interventions resulted in nineteen common behavioral responses that were then sorted by factor analysis and principal components analysis to find common behavioral combinations. The result was three temperament or personality dimensions, which were labeled activity, reactivity, and avoidance for their common characteristics. Positions of individuals on these dimensions were stable across time and surprisingly similar to dimensions found both in humans (Buss and Plomin 1986) and across many animal species (Gosling 1999).

Follow-up studies on the development of temperament in another species, using 37 individuals of *Octopus bimaculatus* Verrill, 1883, were even more interesting. Sinn *et al.* (2001) followed the development of individual differences through the first nine weeks of octopuses' lives, a significant period, as their life span is one year. Fifteen common behaviors were used in the analysis and four factors (Arousal/Readiness, Active Engagement, Aggression and Avoidance/Disinterest) were isolated. As expected if inheritance had a significant effect on temperament, the developmental trajectories of octopuses from the same brood (at least 50% related to one another) were similar. Behavior still showed clear changes across the study, regardless of brood membership. No experimental manipulation was done to alter the environment, as the tiny animals were each kept in a small, barren chamber. A fascinating study would be to see what a stressful early life would do to form the developing personality of the young octopus.

A third study of cephalopod personalities (Sinn and Moltschaniwskyj 2005) used the sepiolid squid *Euprymna tasmanica* Pfeffer, 1884, which has the advantages of having a short five to eight month life span, small size, and solitary habit. This species had four enduring traits: Shy/Boldness, Activity, Reactivity, and Burying Persistence (bury into the soft sand substrate to avoid predation). Unlike in the octopuses, these traits were situation-specific so that activity in the threat test, for instance, did not correlate with that in the feeding one. Also, sex did not affect personality scores although maturity stage did. Fully mature squid were more Threat Active and more Threat Bold as well as less Feed Reactive. This change, in a semelparous species, may reflect a switch from a focus on somatic growth to a short lived concentration on reproduction (Rocha *et al.* 2001). Behaviors in antipredator situations were heritable, while those in feeding ones were not (Sinn *et al.* 2006), and female boldness in foraging explained a small but significant amount of variation in brood hatching success. The convergent results with the studies on octopuses support the idea that relatively stable dimensions of personality may be a characteristic of the cephalopod group.

Play

Many animals react to their environment not by simplistic responses to conditioned stimuli behavior but by exploration, which involves active extraction of information from the surroundings (Hutt 1966). Given a complex environment, many animals will explore and then, as stimuli are repeatedly presented, will habituate to them (Baldwin and Baldwin 1986) and cease to respond. Yet sometimes an animal will instead turn to interactions that are more wide-ranging; Hutt (1966) suggested that orientation changes from 'what does this object do' to 'what can I do with this object?' Play is often defined as simple behavior having no immediate benefits, including repetitive or exaggerated interactions out of sequence compared to normal activity (Burghardt 2004). Play has been considered a human characteristic but is also present in many, though not all, large-brained mammal species (Iwaniuk *et al.* 2001) and sometimes in birds (Diamond and Bond 2003) and is useful for acquisition of adult behavior by altricial young in the protective care of their parents. So why should octopuses play? Possibly because they have manipulative, flexible arms (Kier and Smith 1985, Mather 1998) and thus researchers can recognize and categorize their actions and trace brain capacity for learning (Wells 1978) about the complex subtidal environment in which many octopus species live.

Object play is thought to be expressed when an animal is safe from the danger of predation and when items can be manipulated. We gave *Enteroctopus dofleini* (Wülker, 1910) at the Seattle Aquarium, empty pill bottles weighted to float just at the air-water interface (Mather and Anderson 1999). The tank included a slow water current. Eight octopuses were given ten trials, each lasting until the animal made no contact with the bottle for 30 minutes. Octopuses habituated within trials, spending less and less time in contact with the pill bottle. However, across trials, the situation was quite different as latency to contact and duration of contact with the stimulus did not decrease. Possible play with the object occurred in two of the animals. Each octopus jetted water at the floating bottle until it passed to the far end of the tank and waited until the current returned it to repeat the activity, which resembled bouncing a ball, over 20 circuits in each case. This behavior was different from repulsing the jar, by holding it away with 1-2 suckers on an extended arm.

The form this play behavior took was surprising, causing me to ask why there was not manipulation with the arms? One possibility is that the arms, with 2/3 of the animals' neurons, are under local rather than central control (Rowell 1963, 1966) and that information about the output they produce is not centrally monitored. Local control of pre-programmed responses (*e.g.*, an autotomized arm can walk) might make it more difficult for the arms to produce playful behavior. Another possibility is that the control of the water jet output, which originates from the circulatory system and is used for respiration, is more available for shifts in behavior. Octopuses use jet propulsion for swimming through the water (Wells 1990), though not with the efficiency of the open-ocean squid (O'Dor and Webber 1986). The octopus jet is also used to clean out potential homes (Mather 1994), to excavate clams from the sand (High 1976), and to repel scavenging fish from the remains of prey in the midden outside the home (Mather 1992). This flexibility represents an important characteristic of higher non-stereotyped behavior, defined as using a behavior in a quite different situation (Hirschfeld and Gelman 1994). Perhaps jetting was a behavior that was simply available.

Further investigation of play-like behavior suggested it is a wider phenomenon in octopuses. Kuba *et al.* (2006) tested *Octopus vulgaris* Cuvier, 1797 with presentations of plastic blocks, clam prey, and empty clam shells. The octopuses ate the clams, ignored the empty shells, and sometimes engaged in play-like behavior with the blocks. The play behavior consisted of passing the block from arm to arm, extending the arm and pulling it back near the body, and pulling the block along as the octopus moved. These were designated only play-like on the basis of numbers of repetitions. The arms were clearly central to the actions, and the peak of playful behavior came during the sixth of ten trials, after which the octopuses habituated again. Interestingly, Kuba *et al.* (2006) found that, unlike in mammals (Fagen 1981, Power 2000), young and adult octopuses played the same amount. For the solitary octopus, play was not the result of needing to learn the nuances of a social group, nor was it restricted to the protected environment of the family. It did not occur frequently but occurred equally at different times in the lifespan.

Was this an exception, or will playful behavior be confined to the relatively large-brained and exploratory coleoid cephalopods, perhaps just to the octopuses with their large repertoire of arm actions (Mather 1998)? Do animals have to have a large brain to play? Anecdotal evidence of playful behavior in invertebrates includes snails rising in an aquarium holding on to bubbles, then sinking to the bottom and rising again (Burghardt 2004). One reason we were able to identify play in the octopuses was its similarity to behaviors that playful children perform (*e.g.*, bouncing a ball). Knowledge of the behavioral repertoire of most molluscs is so limited that out-of-sequence and fragmented behavior would usually go unrecognized. How could researchers discriminate play of sea hares, scallops, or nautiloids? Perhaps this is why it has been characterized only in an octopus.

Thinking

Outside of the reflexive behaviors expected of invertebrates lies the whole area of learning, including concept formation, problem solving, and thinking, once thought the domain of primates but now identified in different species, contexts, and adaptive situations (Bekoff *et al.* 2002), including for corvid birds (Emery and Clayton 2004). With capacity for learning from visual and chemotactile stimuli (Wells 1978, Mather 1995), octopuses seem to have the potential for such types of cognitive ability. Yet, in standard indices of accomplishment like Thomas' (1980) levels of learning, octopuses have not shown high scores. Using visual discrimination, octopuses perform reversal learning (Mackintosh and Mackintosh 1963) and attain Thomas' (1980) Level 5, but did not learn the concept of oddity (Boal 1991) to accomplish Level 6. However, ecological constraints rather than learning capacity may be impeding them. Octopuses have great difficulty sustaining the criterion of eight of ten correct responses in continued trials (Papini and Bitterman

1991) used for vertebrates. This may be adaptive since field studies (Mather 1991a) show that they are "win-switch" foragers (Stephens and Krebs 1986), and sampling the unrewarded stimulus or area is useful rather than maladaptive. When a crab is removed from under a rock or a clam dug up from the sand, another will not reappear the next hour or day, and the octopus needs to explore different areas. More ecologically meaningful situations, such as using spatial memory for navigation (Boal *et al.* 2000) as has been demonstrated in their natural environment (Mather 1991b), may be a better test for octopod cognitive capacity.

Nevertheless, there are laboratory situations where octopuses show concept formation, if a concept is defined as abstractions that make it possible for animals to solve novel choice problems without prior experience of the specific exemplars offered (Gould 2002: 43). In a series of experiments on visual shape recognition, Sutherland (1963, also see Wells 1978) hoped to understand what he called the rule of shape discrimination by the octopus visual system, using paired stimuli where the octopus was given a reward for touching one and punishment when touching the other. He found that octopuses could discriminate vertical vs. horizontal extensions of the shape, as in mammals (Matlin and Foley 1997), which are also less competent at discriminating oblique orientations. However, octopuses could also use another rule to discriminate a figure with the same extent on these dimensions but differing in edge-to-area ratio. They could tell a square figure from a circular one of the same dimensions, perhaps by angular changes, and could discriminate the same figure rotated 90 degrees. Sutherland extended six hypotheses about what stimulus dimension the octopus was using to evaluate a figure, but Muntz (1970) produced figures that octopuses could discriminate that did not differ on any one of these. In short, octopuses did not have a single, simple rule for encoding visual shapes but instead chose the correct one for each test. This ability was also shown in Messenger and Sanders' (1972) study, where octopuses trained with two valid cues discriminated faster than those given only one. Octopuses using one cue took longer to transfer to a situation where the other was the relevant one (Mackintosh and Mackintosh 1963). They were learning what aspects of the stimulus were important.

With a variety of techniques to penetrate clam shells, octopuses may also show simple concept formation using chemotactile cues. They use simple trial-and-error learning for the appropriate penetration method (McQuaid 1994, Fiorito and Gherardi 1999, Steer and Semmens 2003), trying to pull the clam valves apart and then switching to the more time-consuming drilling through the shell if necessary. Anderson and Mather (2007) noted that *Enteroctopus dofleini* used different tactics on different clam species, pulling apart the weaker manila clams, breaking the fragile mussels, and drilling or chipping the valve edge of the stronger little neck clams. When manila clams were wired shut, octopuses switched to drilling or chipping. Given intact clams, they ate least of the little neck clams but they preferred this species on the half shell; the excess effort to open the shell overrode their prey preference. Different areas of the clam valves were contacted for each technique, so the octopuses must have switched shell orientation from umbo-to-the-mouth for arm pulling to a lateral presentation for salivary papilla drilling (which was preferentially over the adductor muscles or the heart) or anterior or posterior-to-beak for chipping. Three techniques using three different effectors (arms, salivary papilla, or beak), and three prey positions were all used in the correct combination without access to visual information. Without observation inside the arm web, it is difficult to know if this is simple trial-and-error learning, but the combinations indicate that it is more.

Are such manipulations of information within the capacity of only the coleoid cephalopods within the molluscs? In general, other molluscs have been studied as if such capacities did not exist, and there is excellent information about reflexive behavior and its neural control in the sea hare, scallop and sea slug, for instance. However, Chase (2002) provides some information about the complexity of gastropod behavior that would be an excellent place to start. Owl limpets defend territories on the rocks (Stimson 1970), and other limpets occupy scars for home sites fitted to their own shell for up to three years (Hodgson 1999). How is this behavior controlled? Sea hares migrate long distances (Hamilton 1985), under control of what stimuli? Several gastropods have complex escape responses triggered by the saponin in seastar tube feet (Bullock 1953) and no extensive study of this behavior has been carried out. The authors of chapters in Prete's (2004) book explore examples of flexible, adaptive behavior in other invertebrates, such as color vision of honeybees, prey capture in spiders, and visual recognition in mantis shrimp. Animals can do a lot with simple nervous systems, and molluscs are no doubt among them.

Consciousness

The set of behavioral traits described above suggest that octopuses have a simple form of consciousness. Primary consciousness can be defined as "a reportable multimodal scene composed of perceptual and motor events" (Seth *et al.* 2005: 120) and is sought for in non-human animals. Of course, some theorists argue that no such emergent systems exist, and Gould and Gould (1994) caution against assuming complex cognition where a chain of simple behaviors might be the cause. However, others have suggested that if humans have higher-order consciousness, then homology indicates that non-human vertebrates might have a simpler form. Evidence might be neural, particularly reentrant connectivity between brain areas for perception and those involved in memory (Edelman *et al.* 2005: 170) and to a lesser extent in behavior suggesting such connectivity. As cephalopod brain physiology is still poorly known (but see Williamson and Chrachri 2004 and Hochner *et al.* 2006 for suggestions of such feedback circuits), behavioral evidence must be used primarily for evidence of simple consciousness in these animals (Mather 2007).

Such primary consciousness would be the result of an emergent central representation of the world and oneself. Shepherd (2001) discusses humans' perceptual representation of the external world, although he comments it cannot be a completely accurate one, and Gray (2004) notes that our perceptual world is largely a construct. In fact, one of the basic lessons of human perception is that we form constancies (of color, lightness, and shape) which transcend the immediately available information but make the changing world intelligible to us (Matlin and Foley 1997). Sutherland's studies suggest that octopuses were building such constancies about visual shapes, though none of the early learning studies ever used a comparison that might address this question.

Another important aspect of consciousness is that awareness is only extended to a small proportion of the information incoming from perception and outgoing to muscle control (Gray 2004). Gould and Gould (1994: 149) comment that "thinking is a potentially dangerous backup strategy, too slow and error-prone to be applied indiscriminately." Merker (2005: 98) comments on thinking and assumes its limited task is "optimizing behavioral choices in the light of diverse types of information." One area of information that researchers have assumed is not centrally calculated in octopuses and thus omitted from consciousness, as in humans, is arm movement. To control the muscular, hydrostatic skeletal system (Kier and Smith 1985), octopuses have ganglia all along the arms and above each sucker, and arm control has long been assumed to be reflexive (Rowell 1963, 1966). Recent studies (Sumbre *et al.* 2001, 2006) have confirmed arm control uses peripheral motor programs and simple output strategies in octopus arm extension. Yet Grasso's (2008) studies of the diversity of programming of sucker use challenge this view. Is such flexibility a result of the larger, more complex on-board computer represented by the arm neurons, or is central monitoring evaluating task demands and planning actions? Our mammalian heritage leads us to think of central planning as complex and peripheral as simple, but these divisions may need re-thinking for octopuses.

Baars' (1994) global workspace model of consciousness, with a short-term, attentional spotlight lasting less than a minute, might be a good model for primary consciousness in cephalopods. Merker (2005) feels that such a capacity might arise only in mobile animals with centralized brains faced with decision making in a complex environment. The limited evidence we have suggests that cephalopods do use this kind of decision making; they are active in exploration of their environment (Mather 1991a) and of novel objects introduced to them. For example, Wells (1978) reported that the 'life span' of a floating thermometer introduced into an octopus tank was 5 minutes, and there is anecdotal evidence of similar manipulations noted by aquarium keepers. Octopuses also quickly habituate to the repeated presence of a simple object and move the attentional spotlight except when actions are transformed to play (Mather and Anderson 1999, Kuba *et al.* 2006). Such a central processor could also make decisions about prey entry techniques of octopuses for clams, since action, effector, and clam orientation all must be coordinated (Anderson and Mather 2007)

How might cephalopods' possession of personalities indicate simple consciousness? Merker (2005: 93) assumes that consciousness mediates between motivational, sensory, and motoric functions. The presence of distinct personalities in cephalopods (Mather and Anderson 1993) suggests both motivational differences among individuals and a complex base on which they act. Simple neural structures should produce more behavior that is stereotyped among individuals in the input-output relationships than what is seen in cephalopods. West-Eberhard's (2003) detailed evaluation makes it clear how adaptive plasticity allows combining of genetic influence with environmental pressure (though she had no concept of a central controller) to produce individuals, each adapted over time to its micro-environment.

The ideas advanced in this paper and a deeper evaluation of behavior *per se* can indeed take the study of molluscs in directions it has not traditionally gone. There is much to be gained by doing so: definitely a better understanding of the cephalopods, a new respect for the competence of lowly invertebrates, and new questions to ask, by extension, of all the mollusc groups.

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Freshwater snails (Mollusca: Gastropoda) from the Commonwealth of Dominica with a discussion of their roles in the transmission of parasites

Will K. Reeves[1], Robert T. Dillon, Jr.[2], and Gregory A. Dasch[3]

[1] USDA Agricultural Research Service, Arthropod-Borne Diseases Research Laboratory, Agriculture Building, Room 5031, Department 3354, 1000 E University Avenue, Laramie, Wyoming 82071-2000, U.S.A., wreeves@alumni.clemson.edu

[2] Department of Biology, College of Charleston, Charleston, South Carolina 29424, U.S.A., dillonr@cofc.edu

[3] Centers for Disease Control and Prevention, 1600 Clifton Road NE, MS G-13, Atlanta, Georgia 30333, U.S.A.

Abstract: We collected six species of freshwater snails from Dominica, including *Biomphalaria kuhniana* (Clessin, 1883), *Gundlachia radiata* (Guilding, 1828), *Helisoma* (=*Planorbella*) *trivolvis* (Say, 1817), *Melanoides tuberculata* (Müller, 1774), *Neritina punctulata* Lamarck, 1816, and *Physa marmorata* Guilding, 1828. Our collections indicate that un-reported species such as *G. radiata* and *H. trivolvis* are established on Dominica, West Indies. We tested a limited number of *M. tuberculata* for rickettsial pathogens, *Neorickettsia* spp., but did not identify this agent. Three species of snails previously reported from Dominica, *Biomphalaria glabrata* (Say, 1818), *Biomphalaria straminea* (Dunker, 1848), and *Thiara granifera* (Lamarck, 1822), were not collected. Our data suggest that *B. glabrata* has not re-emerged as a prominent component of the freshwater snail fauna since it disappeared or was locally eradicated. In addition, previous reports of *B. straminea* were probably misidentifications of *B. kuhniana*, and some abnormally large specimens of *M. tuberculata* from Freshwater Lake could be misidentified as *T. granifera*. Our sampling was not adequate to demonstrate that *T. granifera* was absent from Dominica. We determined that *B. kuhniana* was not eradicated by previous molluscan control regimes. Additional studies on the relationships of freshwater snails in Dominica to helminths of animals and humans are needed to understand the public and veterinary health significance of these snails.

Key words: *Biomphalaria, Gundlachia, Helisoma, Physa,* West Indies

The Commonwealth of Dominica is a small (790 km^2) mountainous island nation in the West Indies that receives over 900 cm of rain per year (Grell 1976). The freshwater snail fauna of Dominica has been studied in regard to its significance in the transmission of schistosomiasis (*e.g.*, Noblet and Damian 1991), but the snail fauna has been largely ignored in other regards. Freshwater snails are the primary intermediate hosts for most trematodes, some nematodes, and some rickettsial pathogens (*Neorickettsia* spp.). There have been no reports of autochthonous schistosomiasis on Dominica, but visitors and immigrants harboring the worm have been documented (Grell 1976, Prentice 1980, Grell *et al.* 1981, Noblet and Damian 1991, Adedayo and Nasiiro 2004). There remains a potential for transmission and establishment of schistosomiasis as long as susceptible populations of *Biomphalaria* Preston, 1910 are established on the island. *Biomphalaria glabrata* (Say, 1818) was reported on Dominica (Prentice 1980) but more recent surveys (Noblet and Damian 1991) indicate that this snail was replaced by *Biomphalaria straminea* (Dunker, 1848). In addition, two molluscan intermediate hosts of the trematode *Paragonimus westermani* were introduced on Dominica (Noblet and Damian 1991). The potential to establish this trematode is relatively small because it is not established on neighboring islands. Prosobranch molluscs are the intermediate hosts for trematodes that transmit *Neorickettsia* spp. to humans and domestic animals throughout the Americas (Pusterla *et al.* 2000, Headley *et al.* 2004). *Neorickettsia* spp. have not been reported from Dominica. We conducted a survey of freshwater ponds, lakes, and rivers to determine the distribution of freshwater snails on Dominica. We tested selected *Melanoides tuberculata* (Müller, 1774) for the presence of *Neorickettsia* by PCR amplification of the 16S rRNA gene of *Neorickettsia*.

MATERIALS AND METHODS

Snails were collected (sites listed in Table 1) by removing them from vegetation and mud using nets and sieves or by snorkeling in streams and removing them from rocks. All specimens were preserved in 99% ethanol, which was changed completely after 24 hours.

All snails were identified with morphological characters. DNA was extracted from individual specimens in the genus *Biomphalaria* and 9 *Melanoides tuberculata* from each collection locality. The DNA extraction, PCR, PCR cleanup, and sequencing followed the techniques described by Reeves *et al.* (2006) with the following modifications. We extracted DNA from individual snails and amplified the internal transcribed spacer 2 (ITS-2) and a portion of the 28S rRNA gene from each specimen of *Biomphalaria*, using the primers de-

Table 1. Collection data and snail species identified from sites in Dominica, West Indies in 2005.

Collection site	Habitat type	Collection date	Species collected
Springfield Estate, Parish of Sainte Paul	Pond and outflow stream	12-17 May 2005	*Biomphalaria kuhniana* *Melanoides tuberculata* *Neritinia punctulata* *Physa marmorata*
Roseau Botanic Garden, Roseau, Parish of Sainte George	Artificial pond	13 May 2005	*B. kuhniana* *Helisoma trivolvis* *M. tuberculata* *P. marmorata*
Roseau, open sewer drains, Parish of Sainte George	Standing water	13 May 2005	*B. kuhniana* *P. marmorata*
Roseau River, Roseau, Parish of Sainte George	River	13 May 2005	*N. punctulata*
Clark Hall River, Parish of Saint Paul	River	14 May 2005	*Gundlachia radiata* *M. tuberculata* *N. punctulata*
Freshwater Lake, Parish of Saint George	Lake	15 May 2005	*B. kuhniana* *M. tuberculata* *P. marmorata*
Miranda's Pond, Parish of Saint George	Pond	16 May 2005	*B. kuhniana* *N. punctulata*
Cochran, unnamed pond, Parish of Sainte Paul	Pond	16 May 2005	*G. radiata*
Middleham Falls, Parish of Sainte Paul	River	16 May 2005	*N. punctulata*

scribed by Caldeira *et al.* (2004). DNA extracts from *M. tuberculata* were screened for DNA of *Neorickettsia* by PCR using the EHR16SD and EHR16SR PCR primers to amplify the 16S rRNA gene of *Neorickettsia* as described by Inokuma *et al.* (2000). Positive controls with DNA from *Helisoma trivolvis* (Say, 1817) or *Wolbachia* sp. and a negative control with distilled water were used. We used positive controls that could be amplified by PCR but represented organisms other than those examined in our study.

Voucher specimens of snails were deposited in the Academy of Natural Sciences of Philadelphia. DNA sequences for the ITS-2 and a portion of the 28S rRNA gene of *Biomphalaria kuhniana* (Clessin, 1883) (GenBank Accession #DQ111952) were deposited in GenBank.

RESULTS AND DISCUSSION

We did not collect *Biomphalaria glabrata* or *Biomphalaria straminea* but did collect *Biomphalaria kuhniana* from isolated ponds and Freshwater Lake (Table 1). The DNA sequences for the ITS-2 and a portion of the 28S rRNA gene from our specimens were 100% identical to those of *B. kuhniana* (GenBank #s AY030380, AY030378, AY030379) from Columbia, Dominica, and Venezuela. *Biomphalaria kuhniana* was described from a "Chinese well" in Panama and is morphologically similar to *B. straminea* (Paraense 2003). The two species can be separated with molecular and morphological characters. *Biomphalaria kuhniana* is not a competent intermediate host for *Schistosoma mansoni* (Paraense 2003), which could explain why schistosomiasis has not become established on Dominica even though *Biomphalaria* and occasional transient human infections with *S. mansoni* have been reported in Dominica. Noblet and Damian (1991) reported populations of *B. straminea* in artificial ponds on the island but not from Freshwater Lake. However, we suggest that the previous reports of *B. straminea* were misidentifications of *B. kuhniana*, which was not included in the key by Malek (1985), used to diagnose snails in the previous surveys. In addition we collected *B. kuhniana* in Freshwater Lake, which is a new locality for this snail. DeJong *et al.* (2001) had reported a population of *B. kuhniana* from Roseau, Dominica. *Biomphalaria kuhniana* is not known to serve as the intermediate host of trematodes parasitic to humans or domestic animals; however, little research has focused on the possibility that this snail is a host to helminths other than *S. mansoni*. Other species of *Biomphalaria* are intermediate hosts to echinostomatid trematodes and nematodes in the genus *Angiostrongylus* (Malek 1980).

We collected ancylid limpets in both streams and ponds.

All limpets were morphologically identified as *Gundlachia radiata* (Guilding, 1828), which has not been previously reported from Dominica, but is known from neighboring islands (Starmuhler 1984, Malek 1986). *Gundlachia radiata* is not considered an intermediate host to trematodes of medical significance. It does harbor anisakid nematodes, which are parasitic to fish and some fish-eating mammals (Thiengo *et al.* 2000). Ancylidae are small and often go unnoticed by collectors. These limpets might play important roles in the natural cycles of helminths in Dominica but are currently unstudied.

Helisoma trivolvis (Say, 1817), a planorbid snail that could be mistaken for *Biomphalaria* by untrained collectors, was collected in the metal ponds at the Roseau Botanic Garden. *Helisoma trivolvis* is established in the Dominican Republic and possibly Haiti and Cuba (Ayvazian and Mallett 1986, Paraense 2003). A congeneric species, *Helisoma duryi* (Wetherby, 1879) is also established in the Caribbean (*e.g.*, Pointier 2001). *Helisoma trivolvis* is naturally resistant to infection by *Schistosoma mansoni* (Ayvazian and Mallett 1986), but this snail is an intermediate host to clinostomatid, cyclocoeliid, echinostomatid, and strigeid trematodes and is used as a laboratory host to nematodes in the genus *Angiostrongylus* (Malek 1980, Ponder and Fried 2004). Humans and domestic animals can be infected by some of these worms, including *Alaria canis*, which can cause fatal infections in humans (Malek 1980). As with other zoonotic trematodes, infections of humans are accidental and usually involve eating uncooked meat harboring metacercariae.

We did not collect *Thiara granifera* (Lamarck, 1822), but the specimens of *Melanoides tuberculata* from Freshwater Lake were abnormally large and were initially misidentified as *T. granifera*. *Melanoides tuberculata* was collected in both streams and ponds. We did not amplify DNA from *Neorickettsia* spp. in any of our collections of *M. tuberculata*. This exotic snail was possibly introduced to Dominica around 1975 (Pointier and McCullough 1989). *Melanoides tuberculata* is a potential biological control agent for *Biomphalaria* spp. because the two snails appear to compete, and *M. tuberculata* might exclude *Biomphalaria* spp. in some habitats (Pointier and McCullough 1989). Our data indicate that exclusion does not occur in Dominica (Table 1). *Melanoides tuberculata* is not a suitable host for *Schistosoma mansoni* but is an intermediate host to *Paragonimus westermani*, a lung fluke. There is a possibility that *P. westermani* or other *Paragonimus* spp. will become established on Dominica, because both *M. tuberculata* and the freshwater-crab, second-intermediate hosts of *P. westermani*, are present (Noblet and Damian 1991). Carnivorous mammals are natural hosts for this fluke so zoonotic cycles of the parasite could become established without human infections. *Melanoides tuberculata* can serve as the intermediate host to other trematodes that occasionally infect humans, including eye flukes (*Philophthalmus* spp.) of birds (Dimitrov *et al.* 2000, Lamothe-Argumendo *et al.* 2003). *Melanoides tuberculata* is also an intermediate host to *Heterophyes heterophyes*, a fluke of fish-eating mammals and birds (Malek 1980). *Heterophyes heterophyes* can infect humans.

Physa marmorata Guilding, 1828 was collected from ponds and water tanks with freshwater plants. Physid snails are often overlooked as intermediate hosts of helminths, but *P. marmorata* is the intermediate host for the echinostomatid trematodes, *Echinostoma luisreyi* and *Echinostoma paraensei* (Maldonado *et al.* 2001, 2003). *Physa* spp. are intermediate hosts for diplostomatid, echinostomatid, and strigeid trematodes and are laboratory hosts for nematodes in the genus *Angiostrongylus* (Malek 1980). In addition, *Physa* spp. serve as hosts to nematomorph worms and the oligochaete *Chaetogaster* sp., which are parasites of invertebrates (Gamble and Fried 1976, Hanelt *et al.* 2001). The public health or veterinary significance of populations of *P. marmorata* on Dominica are unknown, but further studies could prove this snail a host to helminths of economic significance. Noblet and Damian (1991) reported collecting *Physa cubensis* Pfeiffer, 1839 in Dominica. *Physa cubensis* is a junior synonym of *Physa acuta* Draparnaud, 1805 (Paraense and Pointier 2003). However, we collected *P. marmorata* in the same habitats and localities that Noblet and Damian (1991) reported *P. acuta*. These older reports of *P. acuta* thus probably represent misidentifications of *P. marmorata*.

The neritid snail *Neritina punctulata* Lamarck, 1816 was collected from both streams and fish ponds. *Neritina punctulata* occurs in streams throughout Dominica (Starmuhlner 1984) and breeds in streams with eggs attached to boulders. *Neritina punctulata* is possibly the largest freshwater snail on Dominica and is not known to harbor parasites of humans or domestic animals. However, this snail is eaten by humans on Dominica. As long as the snails are adequately cooked, they would pose no threat even if they were intermediate hosts to helminths or other infectious agents. A detailed study of the potential helminths or other infectious agents pathogenic to humans in *N. punctulata* will thus have public health implications.

Exotic snails continue to be introduced into Dominica. There is at least one tropical fish store on the island, and tropical ampullarids, physids, and planorbids are sold by the tropical fish industry. Freshwater plants such as water lettuce (*Pistia stratiotes*) are transported from one Caribbean Island to another by travelers and are used as ornamental plants in local water gardens or fishponds. Snails such as *Biomphalaria* spp. can be transported on these plants.

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Patterns of activity cycles in juvenile California two-spot octopuses (*Octopus bimaculoides*)

David L. Sinn

School of Zoology, University of Tasmania, Private Bag 5, Hobart, Tasmania 7001, Australia, david.sinn@utas.edu.au

Abstract: Octopuses function as important prey and predators in many continental-shelf marine ecosystems. Understanding activity cycles of octopuses should help define their mode of foraging and potential resource utilization and, therefore, their niche within the marine community. Unfortunately, little is known concerning activity cycles of octopuses, especially during their juvenile life-history stages. Here, I present observations on juvenile activity in *Octopus bimaculoides* Pickford and McConnaughey, 1949 over three observational weeks in a semi-natural laboratory setting. Octopuses on average were nocturnal, but some individuals were active during daylight hours in all three observational weeks. Nocturnal activity cycles may decrease the risk of predation on juveniles by visual fish predators hunting during daylight hours. However, inter- and intraspecific competition with other octopuses in different life history stages, including adult *O. bimaculoides* and adult and juvenile *Octopus bimaculatus* Verrill, 1883 is also likely during nighttime hours. Further studies are needed on the relative influence of predation and competition on octopus activity cycles and the resulting consequences for octopus populations.

Key words: cephalopod, octopus activity, juvenile ecology, niche partitioning

Activity cycles play an important role in determining a species' niche. At the individual level, an organism's activity cycle mediates the ecological trade-off between growth and mortality (Werner and Anholt 1993). Increased activity can lead to increased resource acquisition (and therefore, growth) but may also come at a cost, by increasing susceptibility to predation. At the level of populations, activity is manifest through levels of intraspecific competition between life history stages (*e.g.*, between juveniles, sub-adults, and adults) or through interspecific competition between sympatric species competing for similar resources (Werner 1992). For example, two species which overlap in feeding or habitat niche can avoid direct competition if their populations exhibit non-overlapping activity cycles.

Octopuses are an important mid-level trophic component of many shallow continental shelf marine ecosystems, as both generalist predators on many smaller fish and invertebrate prey (Fawcett 1984, Ambrose 1986) and as important prey items for larger fish and marine mammals (Hanlon and Messenger 1996, Forsythe and Hanlon 1997). Many of these predator/prey relationships should influence, and be influenced by, the activity cycles of their constituent organisms (*e.g.*, Richardson 2001). For example, fish, octopus, and/or other invertebrate predators could be a major selective force shaping (but also responding to) the general activity cycles of fish, octopus, and/or invertebrate prey populations (Daido 2002). Understanding activity cycles of octopuses at multiple life-history stages (*i.e.*, juvenile, sub-adult, and adult stages) may therefore contribute to our understanding of the structuring of resource use among populations and ecological communities in shallow, near-shore marine environments.

Reports of activity cycles in octopuses are scarce, but several species have been studied and characterized as having a basic endogenous day, night, or crepuscular activity pattern. At least some populations of several species of octopus are nocturnal (*Enteroctopus dofleini* (Wülker, 1910): Hartwick *et al.* 1984; *Octopus joubini* Robson, 1929: Mather 1984; *Octopus macropus* Risso, 1826: Meisel *et al.* 2006), whereas other species show a tendency towards diurnal patterns of activity (*Octopus cyanea* Gray, 1849: Forsythe and Hanlon 1997, Hanlon *et al.* 1999). Some species (*e.g.*, *Octopus vulgaris* Cuvier, 1797) may be characterized by populations or individuals which can be nocturnal, diurnal (Wells *et al.* 1983, Meisel *et al.* 2006), and, under certain conditions, arrhythmic (Wells *et al.* 1983, Meisel *et al.* 2003). Overall, these previous studies illustrate substantial variation within and among species and populations of adult octopuses. Unfortunately, there is little information regarding activity cycles of juvenile octopuses of any species, probably due to their small size and highly cryptic nature in the wild.

Octopus bimaculoides Pickford and McConnaughey, 1949 occurs in shallow continental-shelf regions of central California to Baja California, Mexico, where it inhabits rocky reef, kelp forests, and mudflats (Lang 1997). Currently, most observations of *O. bimaculoides* have come from laboratory studies (Forsythe and Hanlon 1988a, Sinn *et al.* 2001); only one study has reported limited *in situ* observations (Lang 1997). Like most shallow-water octopuses, *O. bimaculoides* has a short lifespan (Forsythe and Hanlon 1988b). Adult female *O. bimaculoides* lay numerous (Forsythe and Hanlon 1988a, 1988b), large teardrop-shaped eggs (3-4 mm long) from which relatively well-developed benthic hatchlings are produced after an incubation period of 6-8 weeks. Upon hatching, juveniles disperse by crawling or swimming (as opposed to solely relying on ocean currents). Little is known about the foraging ecology of juveniles in this species, but in laboratory settings juveniles are generalist feeders and will take small shrimp, crabs, bivalves, fish, and gastropods (Forsythe *et al.* 1984, Sinn 2000). There have been no systematic reports of activity cycles in any life stage of *O. bimaculoides.*

The aim of the current study was to determine whether juvenile *Octopus bimaculoides* displayed patterns of activity cycles which could be described as crepuscular, nocturnal, or diurnal under semi-natural laboratory conditions (*i.e.*, natural day lengths and *ad libitum* feeding).

MATERIALS AND METHODS

Brooding female specimens of *Octopus bimaculoides* were obtained commercially from the wild (Chuck Winkler, Long Beach, California) in September 1998 and shipped to Portland, Oregon, where they were maintained until eggs hatched. Two broods hatched synchronously during the week of October 15, 1998, and the majority of individuals hatched within 3-4 days of this date. Within 2-3 days of hatching, octopuses from these two broods were assigned randomly and in equal numbers to two separate holding tubs where they remained until the end of experiments. Holding tubs were 76-L fiberglass tubs (1 m × ⅓ m × ⅓ m) in-line with a 1900-L closed-seawater system. Salinity (34-36 psu) and temperature (18 °C) were held constant throughout experiments. The system had overhead fluorescent lighting in addition to natural, direct sunlight from a large bank of adjacent windows. To allow for diffuse dawn and dusk periods, the day/night light cycle of the fluorescent lights was timed to come on one hour after sunrise and turned off one hour before sunset. Sunrise/sunset times were based on data for Portland, Oregon from *The Astronomical Almanac Online* (U.S. Naval Observatory and H.M. Nautical Almanac Office; http://asa.usno.navy.mil/). Low-powered red lights (25 W), which were never turned off, allowed observations during nighttime hours. Holding tubs contained crushed oyster shell substrate and plastic seagrass beds along with shelter in the form of clay pots, small PVC tubing, and rocks. Hatchling/juvenile octopuses were fed *ad libitum* during non-observation days with littorinid snails (*Littorina scutulata* Gould, 1849), mysid shrimp (*Mysis* spp.), and small shore crabs (*Hemigrapsus* spp.). Juvenile specimens of *O. bimaculoides* have high mortality rates relative to adults (Forsythe *et al.* 1984). Therefore, in order to enhance survival, an attempt was made to maintain at least one type of food source in tubs at all times.

A single 24-hr long observation period on both tubs of octopuses began on October 29, 1998 (N = 98 octopuses), when animals were approx. 14 days old. Subsequent 24-hr long observation periods were made on November 11, 1998 (N = 88 octopuses) and November 17, 1998 (N = 87 octopuses). For each 24 hr period, counts of 'active' octopuses were made on each tub once per hour at the beginning of each hour, beginning at 5 PM on each date. An octopus was considered 'active' if it was crawling, swimming, or sitting outside of cover (at least 50% of its body). The number of 'inactive' octopuses for each count was calculated by subtracting the number of active octopuses counted from the total number of animals within each tank, which had been assessed the day previous to observations by removing all cover and counting total octopuses. The proportion of octopuses active across both tubs was then calculated by dividing the total number of active animals by the total number of active and inactive animals. This method resulted in twenty-four observations per tub per observation day.

A nocturnal versus diurnal activity cycle was tested by examining the proportion of active individuals during daylight hours (7 AM to 5 PM) versus nighttime ones (5 PM to 7 AM) for each week. To examine whether octopuses were crepuscular, mean proportions of active individuals were compared across two time periods for each week, the first representing dawn/dusk periods (6 AM to 8 AM and 4 PM to 6 PM) and the second period representing all other hours (8 AM to 4 PM and 6 PM to 6 AM). Temporal autocorrelation between observations within a tub and an unbalanced statistical design precluded use of hypothesis tests. For example, activity at one observation period was probably not independent of activity during another, and 6 observation times were taken to compute a mean proportion for dawn/dusk activity while 18 observations were used to compute mean activity during 'all other times'. Thus, for statistical comparisons, the mean proportion of active octopuses for the appropriate time period was calculated for each week and graphed along with 95% confidence intervals. Non-overlapping 95% confidence intervals of means were used as a conservative estimate of statistically significant differences because a comparable statistical test would always indicate a statistically significant difference at $P < 0.05$ for two non-overlapping 95% confidence intervals (Payton *et al.* 2003).

RESULTS

There was little evidence to support a crepuscular activity cycle in juvenile *Octopus bimaculoides.* The mean proportion of octopuses active during dawn/dusk periods and all other time periods was not different for any of the three observation weeks (Fig. 1). Instead, juveniles on average exhibited nocturnal activity cycles, as the mean proportion of

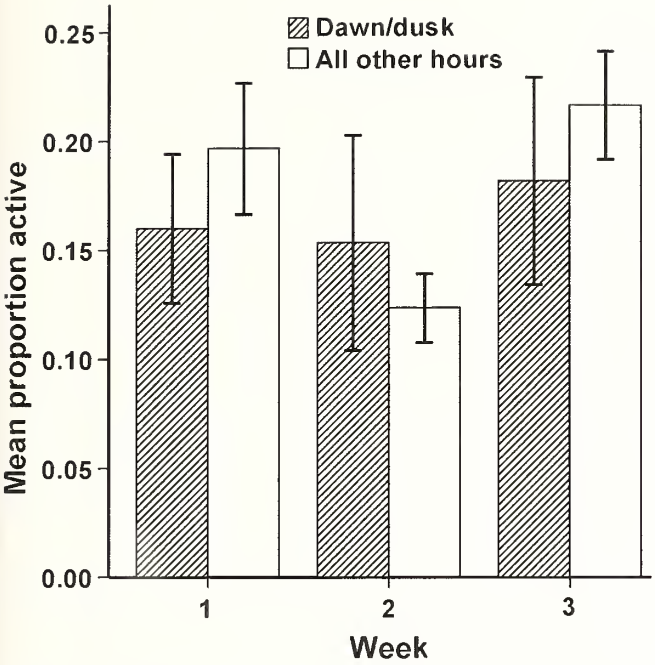

Figure 1. Mean proportion of active juvenile *Octopus bimaculoides* during dawn/dusk (6 AM to 8 AM and 4 PM to 6 PM) versus all other time periods over three weeks. Error bars represent 95% confidence intervals.

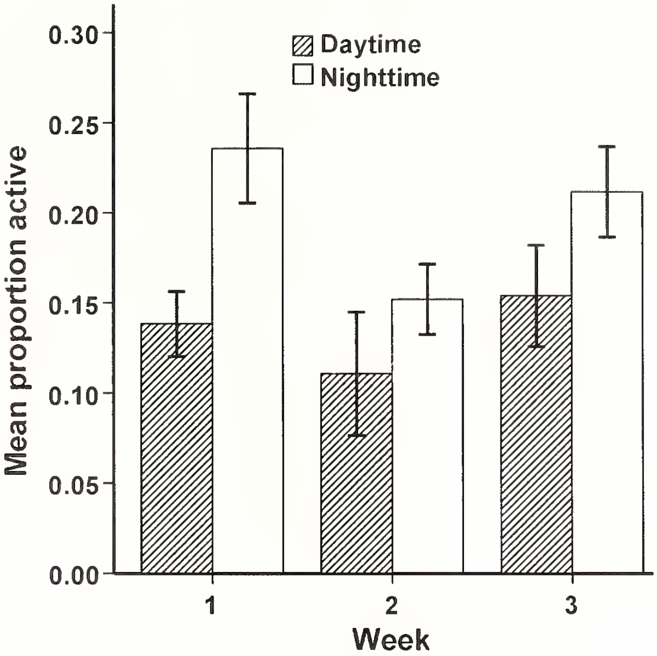

Figure 2. Mean proportion of active juvenile *Octopus bimaculoides* during nighttime hours (5 PM to 7 AM) versus daylight hours (7 AM to 5 PM) over three weeks. Error bars represent 95% confidence intervals.

active juvenile octopuses was greater during nighttime hours than daylight ones in two out of the three weeks (Fig. 2). The activity cycles could be characterized qualitatively each week by an increase in activity which coincided with sunset periods; this activity generally peaked at midnight, after which time there was a steady decrease in activity until the next day's sunset (Fig. 3).

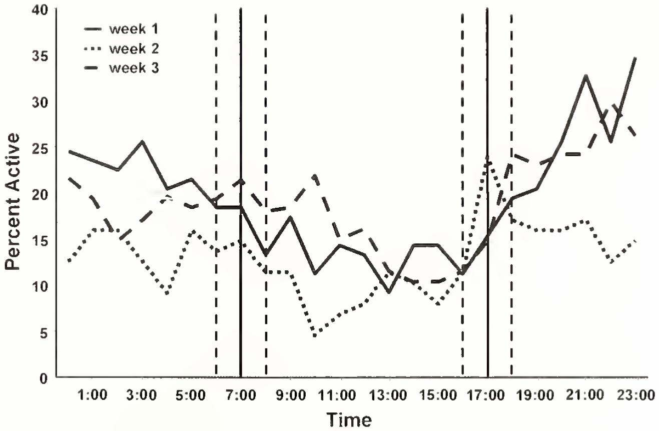

Figure 3. Percentage of juvenile octopuses that was active during each 5-minute monitoring period during each hour. Solid vertical lines represent average sunrise (mean = 7:01 AM, *SD* = 13 min) and sunset (mean = 4:48 PM; *SD* = 12 min). Dashed vertical lines represent dawn (6 to 8 AM) and dusk (4 to 6 PM) periods. Sample sizes varied by week (week 1: *N* = 98; week 2: *N* = 88; week 3: *N* = 87).

DISCUSSION

This is the first report of activity cycles in juvenile California two-spot octopuses, *Octopus bimaculoides*. Under natural daylight conditions and constant food availability, juvenile animals tended to be nocturnal in their activity. This tendency could be potentially adaptive for individuals if predation risk for juvenile octopuses were greater in daylight than nighttime. High numbers of visual predators active during daylight hours (*e.g.*, teleost fish) may favor juvenile octopuses which are active during nighttime hours (Aronson 1991, Hanlon and Messenger 1996). However, intra- and interspecific competition with other octopuses through niche overlap should also influence activity cycles and would favor temporal spacing of time budgets between populations (Houck 1982, Meisel *et al.* 2006). *Octopus bimaculoides* occurs sympatrically along its range with *Octopus bimaculatus*, and these two sister species most likely occupy similar niches (Pickford and McConnaughey 1949). Some populations of adult *O. bimaculatus* can be nocturnal (Ambrose 1982), and nocturnal activity in adult *O. bimaculoides* in the laboratory has also been observed (Sinn 2000). Taken together, these reports suggest that juvenile *O. bimaculoides* may face multiple, conflicting selection pressures influencing their activity patterns. Clearly, further work is needed on the relative influences of predation risk and niche overlap on the activity cycles of juvenile octopuses.

Even in daytime periods, at least some octopuses were active, and during one observation week, there were no differences between activity in daytime and nighttime hours. One explanation could be that metabolic demands for fast-growing juvenile cephalopods are high (Lee 1994), requiring animals to feed throughout a 24-hr cycle. Two other environmental cues which may have influenced the activity cycles of juvenile octopuses, namely food availability and octopus density, are also worth further consideration. For example, food availability influences adult activity in other *Octopus* species, with *ad libitum* feeding resulting in a lack of a discernable 24-hr cycle in adult *Octopus vulgaris* (Wells *et al.* 1983). While constant food availability was chosen here to maximize juvenile survivorship, in the wild, food availability may not be limiting for juvenile octopuses (*e.g.*, *O. bimaculatus*: Ambrose 1988). Second, octopus densities may also have influenced individual activity cycles if densities in tubs were unnaturally high. Increased density may have increased aggressive interactions with conspecifics during peak activity hours (*i.e.*, nighttime) and resulted in some individuals becoming active during daylight (*e.g.*, Sinn *et al.* 2001). Nothing is known concerning natural densities of juvenile *O. bimaculoides*, but adult individuals of *O. bimaculoides* have been reported to spatially group in high densities in suitable habitat in the wild (Lang 1997).

From a practical standpoint, the environmental circumstances experienced by octopuses in the current study were probably similar to culturing conditions other researchers employ when studying juvenile octopuses (*i.e.*, constant food availability to ensure low mortality rates). Understanding the basic activity cycles of laboratory animals is necessary to properly perform experimental manipulations and to recognize 'abnormal' behavior which could indicate sick or dying animals (Moltschaniwskyj *et al.* 2007). Unfortunately, detailed study of cryptic, juvenile octopuses in the wild will probably remain intractable for some time. Therefore, inferences based on laboratory reports, taken with caution, remain the sole information available to understand the interaction between circadian rhythms and the juvenile ecology of many *Octopus* spp.

This study is a first step toward quantifying activity cycles in the juvenile life stages of *Octopus bimaculoides* and provides a baseline for studying juvenile life stages both in the laboratory and field. Understanding the activity cycles of different life-history stages of *Octopus* spp. under natural or semi-natural conditions should contribute to our understanding of the ecological costs and benefits that arise from an animal taking a particular activity strategy under a given set of conditions (Sinn *et al.* 2001). Further work is clearly needed on the influences of competition and predation risk on octopus activity cycles and the resulting population- and species-specific outcomes (Ambrose 1982, 1986).

ACKNOWLEDGMENTS

Chuck Winkler provided female *Octopus bimaculoides* with eggs for this project, and Mark Stapleton helped in data collection. Leonard Simpson and the Department of Biology at Portland State University, Oregon, provided essential laboratory and financial support for this project. Russell Thomson made helpful suggestions with regards to data analysis. Roland Anderson and Jennifer Mather provided constructive criticism on an earlier draft of this manuscript, and two anonymous reviewers made helpful suggestions on a later version.

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Discovery of the South African polyplacophoran *Stenosemus simplicissimus* (Thiele, 1906) (Mollusca, Polyplacophora, Ischnochitonidae) in the Southern Ocean

Enrico Schwabe

Zoologische Staatssammlung München, Münchhausenstrasse 21, D-81247 München, Germany, enrico.schwabe@zsm.mwn.de

Abstract: Recent expeditions to the Atlantic sector of the Southern Ocean have yielded valuable collections of shelf and deep water polyplacophorans. These included several specimens of *Stenosemus simplicissimus* (Thiele, 1906), a species previously known only by its holotype and type locality at the Cape of Good Hope. The new material enabled a thorough morphological redescription of the species by studying valve, perinotum, and radula characters with SEM. The new records from Shag Rocks and the eastern Weddell Sea enlarge the species' biogeographic distribution from the temperate South African region to the polar South Georgia and Weddell Sea regions. Its bathymetric range is extended from 318 m to 285-1064 m. The limited occurrence of deep-water Antarctic polyplacophorans may be caused by benthic predators that limit the expansion of non-herbivorous chitons in the Antarctic deep sea.

Key words: Atlantic Ocean, Antarctica, zoogeography, distribution, new records

Antarctic waters support a small number of Polyplacophora in contrast to the highly diverse fauna of other marine molluscs (Thiele 1912, Dell 1990, Numanami 1996, Sirenko and Schrödl 2001). The species discovered to date are: *Leptochiton kerguelensis* Haddon, 1886, *Callochiton bouveti* Thiele, 1906, *Callochiton gaussae* Thiele, 1908, *Leloupia belgicae* (Pelseneer, 1903), *Stenosemus exaratus* (G. O. Sars, 1878), *Stenosemus simplicissimus* (Thiele, 1906), *Tonicina zschaui* (Pfeffer *in* von Martens and Pfeffer, 1886), *Nuttallochiton mirandus* (E. A. Smith MS, Thiele, 1906), and *Hemiathrum setulosum* Carpenter in Dall, 1876. With the exception of *S. simplicissimus*, all species are more or less well described in earlier revisions (*e.g.*, Thiele 1906a, 1906b, 1908, Dell 1964, Kaas and Van Belle 1985a, 1985b, 1990, Götting 1993). The recent rediscoveries of this species, which was known only from the type material, enable a detailed morphological description using scanning electron microscopy (hereafter, SEM). The description will help non-chiton specialists to separate this species from similar representatives of the genus *Callochiton* Gray, 1847. In addition, deep water polyplacophorans from Antarctica are rare and our knowledge of their biology and habitat preference is limited. Analysis of abiotic parameters at a certain depth may help getting a better understanding of how chitons interact with their environment. The present paper deals with the unknown Antarctic deep-water chiton fauna.

MATERIALS AND METHODS

Specimens of *Stenosemus simplicissimus* were collected during recent expeditions with R/V *Polarstern* (ANT XIII/3, ANT XVII/3, ANT XXI/2, and ANT XXII/3) to the Scotia and eastern Weddell Seas. The material was obtained by using Agassiz trawls (AGT), bottom trawls (BT), epibenthic sledges (EBS), and Rauschert dredges (RD). When the catch reached the deck, the samples were sieved through 500 μm or 1000 μm mesh, the remainder was fixed in 4% buffered formaldehyde or 75-96% ethanol and then sorted under stereomicroscopes. Most of the polyplacophorans were found attached to hard substrates, such as cobbles. Specimens were stored in ethanol for further morphological examinations. To confirm the identification of *S. simplicissimus*, the holotype was examined.

The type material is deposited at the Natural History Museum Berlin, Germany (ZMB). The newly collected material is deposited at the British Antarctic Survey, United Kingdom (BAS), the Zoological Institute St. Petersburg, Russia (ZISP), and the Bavarian State Collection of Zoology, Germany (ZSM).

Preparation of the specimens followed Schwabe and Ruthensteiner (2001). Specimens used for SEM were partly disarticulated, enabling examination of valves, perinotum, and radula. Microsculpture and radular photographs were made on a LEO 1430VP SEM (at the ZSM). Abiotic factors were established using a conductivity-temperature-depth (CTD) data logger, or by visual inspection of the substratum on board of the research vessel.

SYSTEMATICS

Class Polyplacophora Gray, 1821
Subclass Neoloricata Bergenhayn, 1955
Order Chitonida Thiele, 1910
Family Ischnochitonidae Dall, 1889

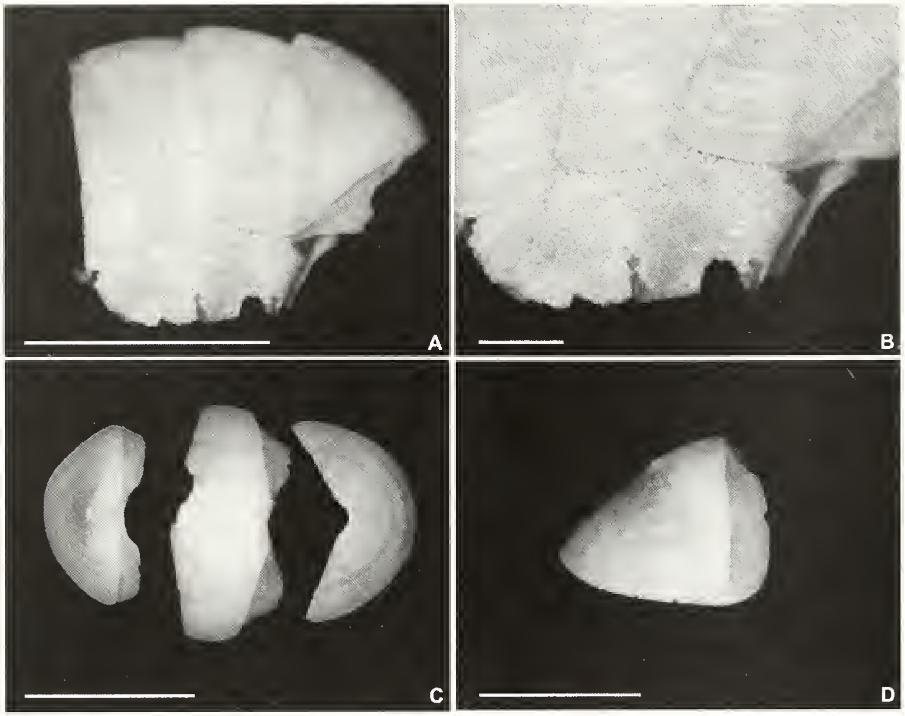

Figure 1. Light micrographs of the holotype of *Stenosemus simplicissimus*, ZMB Moll 59908. A, valves ii to iv *in situ*, anterior at right; B, detail of fig. 1A, showing the dorsal perinotum scales; C, valves (from left to right) viii, v, i in dorsal view, anterior at right; D, right lateral view of the tail valve, anterior at right. Scale bars: A, 5 mm; B, D, 1 mm; C, 500 μm.

Genus *Stenosemus* von Middendorff, 1847
Type species: *Chiton albus* Linnaeus, 1767, by subsequent designation, Winckworth (1926: 15)

Stenosemus simplicissimus (Thiele, 1906)
(Figs. 1-5)

Ischnochiton (Chondropleura) simplicissimus Thiele 1906b: 335, pl. 29, figs 21-25.

Additions to the bibliography in Kaas and Van Belle (1990: 67):

Ischnochiton simplicissimus; Barnard 1974: 740.
Ischnochiton (Stenosemus) simplicissimus; Kaas and Van Belle 1980: 120; 1990: 67, fig. 27; 1998: 171.
Ischnochiton (Chondropleura) simplicissimus; Kilias 1995: 169.
Stenosemus simplicissimus; Sirenko 1994: 164; 2005: 36; Gutt *et al.* 2000: 40; Linse *et al.* 2006: 155 (partim).
Type material: ZMB Moll 59908 (partly disarticulated holotype) (Figs. 1A-D).
Type locality: South Africa, Cape of Good Hope, Deutsche Tiefsee-Expedition St. 113: 34°33.3′S 18°21.2′E, 318 m.

Additional material examined

ZSM Mol 20050857 (1 specimen - 3.4 × 2.2 mm, partly disarticulated) (Figs. 2-3), Antarctica, Weddell Sea: ANT XXII-3 (ANDEEP III) St. PS 67/074-7: 71°18.60′S 13°59.11′W-71°18.38′S 13°58.17′W, 1047-1064 m, on rock (quartz-amphibolite gneiss), laying on a sandy sediment (only 5 cm thick) (salinity: 34.7 psu; water temperature: 0.5 °C; O_2 concentration: 5.8 ml/l; pressure: 1016.5 dBar; all data from 1004 m, measured by CTD), AGT, collected by J. M. Bohn and E. Schwabe, 20 February 2005, preserved in 96% ethanol.

ZSM Mol 20020914 (1 specimen - width 3.1 mm [curled]), Shag Rocks, South Georgia and South Sandwich Islands: ANT XIX-5 (LAMPOS) St. PS 61/169-1: 53°22.94′S 42°41.37′W-53°22.89′S 42°41.50′W, 284.3 m, RD, collected by Dr. M. Schrödl, 10 April 2002, preserved in 78% ethanol.

ZSM Mol 20008502 (1 specimen - 8.7 × 4.2 mm, partly disarticulated) (Fig. 4), Antarctica, Weddell Sea: ANT XVII-3 (EASIZ III) St. 97-1: 71°06.27′S 12°50.46′W-71°06.24′S 12°49.92′W, 728-743 m, EBS, collected by Dr. M. Schrödl, 3 April 2000, preserved in 78% ethanol.

BAS (Dr. Katrin Linse) 03-802 (1 specimen - width 3.8 mm [curled]), Antarctica, Weddell Sea: ANT XXI-2 (BENDEX) St. PS 65/324-1: 72°54.52′S 19°47.74′W-72°54.55′S 19°47.30′W, 647.2-693.6 m, RD, collected by Dr. K. Linse, 3 January 2004, preserved in 96% ethanol.

BAS (Dr. K. Linse) 03-769 (1 specimen - width 3.4 mm [curled]), Antarctica, Weddell Sea: ANT XXI-2 (BENDEX) St. PS 65/297-1: 72°48.50′S 19°31.66′W-72°48.65′S 19°31.85′W, 630.8-668 m, RD, collected by Dr. K. Linse, 1 January 2004, preserved in 96% ethanol.

Description

Species moderately large, up to 16 mm (the largest specimen is the holotype). It is elongate, oval, with a cari-

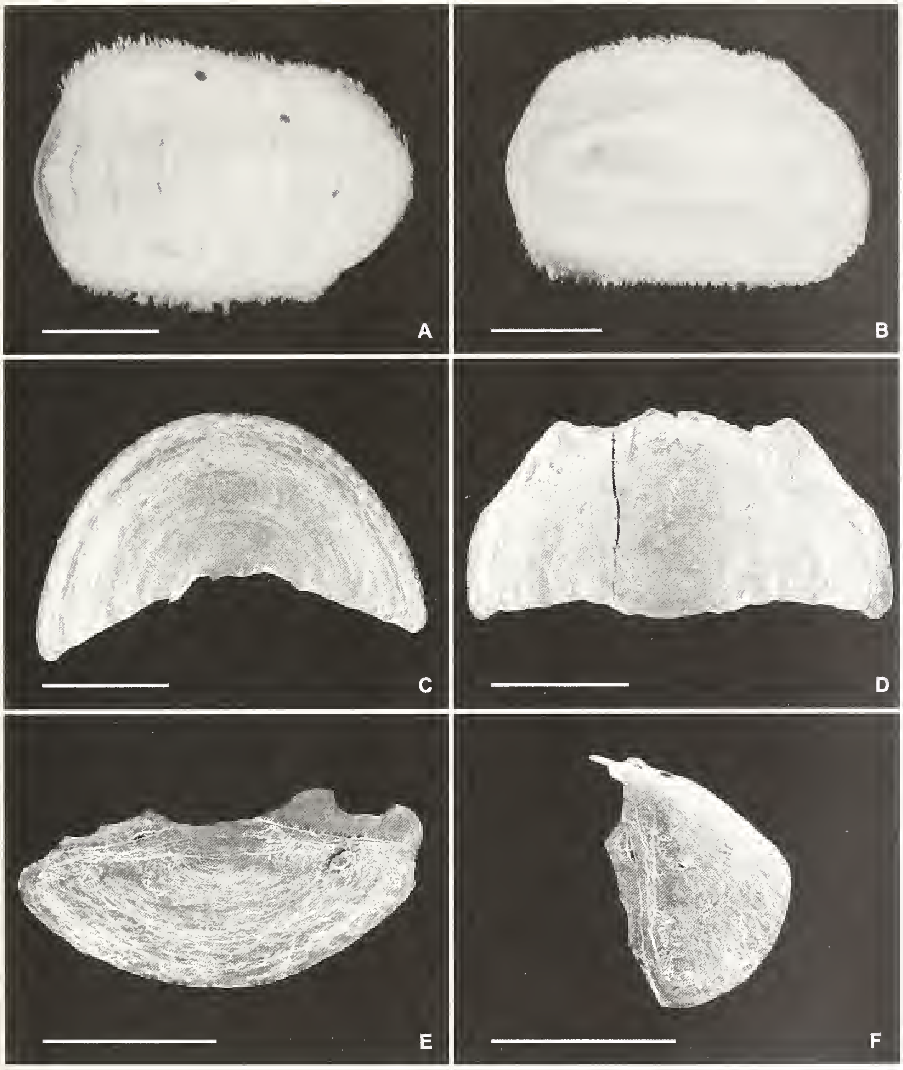

Figure 2. *Stenosemus simplicissimus* (ZSM Mol 20050857), 3.4 × 2.2 mm. A-B, light micrographs, C-F, scanning electron micrographs. A, dorsal view of the complete specimen, anterior at right; B, ventral view of the complete specimen, anterior at right; C, dorsal view of the head valve; D, dorsal view of valve ii; E, dorsal view of the tail valve; F, left lateral view of the tail valve, anterior at left. Scale bars A-B: 1 mm, C-F: 500 µm.

nated, moderately high-elevated dorsum. Dorsal elevation quotient (height/width) (of isolated valve v of the holotype): 0.51. Color of tegmentum and perinotum uniform dull white.

Tegmentum virtually smooth, except for commarginal growth marks, which occur on all valves (Figs. 1A-D, 2C-F, 4A-D) and a micro-perforation. In earlier growth stages, fine radial striation is visible in apical regions and the middle of the first valve. The head valve has a wide V-shaped posterior margin and is clearly notched in the middle (Figs. 1C, 2C, 4A). Intermediate valves (Figs. 1A, 1C, 2D, 4B) are trapezoid (valve ii) to rectangular, with short and rounded side margins, and straight to slightly concave posterior margins (on both sides of the slightly protruding apex). Anterior valve margin is convex. Lateral areas are clearly elevated. Tail valve (Figs. 1C-D, 2E-F, 4C-D) is semicircular with an anteriorly directed, weakly elevated mucro that is situated in the two anterior thirds of the valve length. Postmucronal slope is steep and straight.

Articulamentum is thin and white. Apophyses (Figs. 1A, 1C-D, 2D-E, 4B-D) are well developed, rather short, wide, and medially connected by a short smooth jugal lamina. Apophyses are triangular in intermediate valves, and rectangular in tail valve. Slit formula varies from 14/1/10 (holotype) to 16/1-2/13 (8.7 mm long specimen, ZSM Mol 20008502). Slits are wide and rather long, teeth edges are slightly thickened and faintly crenulated. Slit rays are present in all valves. Eaves are spongy.

Perinotum is rather narrow (Figs. 1A-B), dorsally covered with juxtaposed, bent, conical, round-topped, and inwardly directed calcareous scales, 160-176 µm long, 144-150 µm in diameter on mid-perinotum, rhomboidal at the base, and weakly longitudinally striated (Kaas and Van Belle 1990). Towards the outer margin, the scales are smaller and measure 80-90 × 50-57 µm (Fig. 3B). Marginal fringe (Fig. 3C) consists of straight, obtusely-pointed solid spicules, which are medially keeled and measure about 110 × 22 µm. Among them are smaller, straight, and sharp spicules, 50 µm in length and 10 µm in width. These spicules are situated distally either on long, very slender shafts (140 × 7 µm) or in shorter tubs that may attain a diameter of 15 µm (Fig. 4E). Ventrally there are radial rows of rectangular scales that measure 53 × 27 µm (Fig. 3D).

Radula (Fig. 3D) of a partially disarticulated specimen (ZSM Mol 20008502) measures 3.3 mm, of which 1.6 mm were taken up by the radula cartilage, with 44 teeth rows of which 31 are mineralized. Central tooth is rectangular, its distal end a little wider than the base. It measures 71 × 31 µm long and has a forward-directed simple, slightly bent cusp.

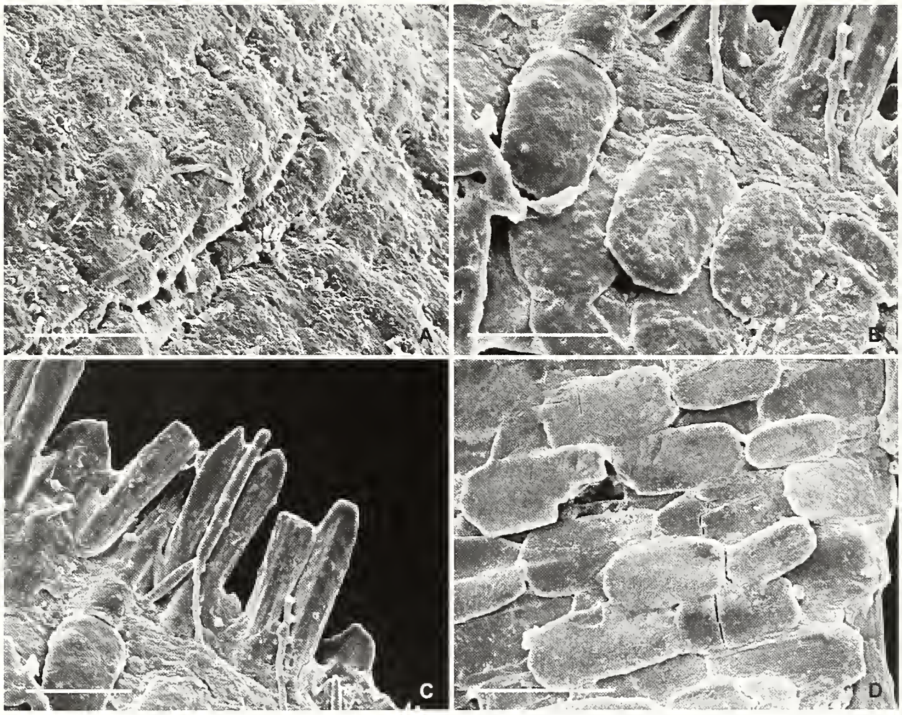

Figure 3. Scanning electron micrographs of *Stenosemus simplicissimus* (ZSM Mol 20050857), 3.4 × 2.2 mm. A, detail of Fig. 2F, showing the valve microsculpture; B, dorsal perinotum scales at the margin; C, marginal perinotum fringe; D, ventral perinotum scales. Scale bars: 50 µm.

First lateral tooth is wing-shaped and covers the lower two thirds of central tooth. It may attain a length of 60 µm and has a simple inward directed small cusp. Total length of second lateral tooth is 175 µm; one third of its length is taken up by the squarish head with the single, sharply pointed, and inwardly curved elongate denticle. Shaft of second lateral tooth is sharply keeled on its inner side and slightly curved in the upper half. Basally the tooth is wing-shaped. First uncinal tooth is triangular in outline with a straight inner edge and a steep slightly convex, outer edge. Second uncinal tooth is very broad and S-shaped. Third uncinal tooth is extremely slender, measuring 138 × 18 µm, with a spoon-shaped distal extension, which may attain the double width of the shaft. First marginal tooth is similar to, but slightly more slender than, the second uncinal. Second marginal tooth is arrowhead-shaped, measuring 71 × 31 µm. Third marginal tooth is rectangular in outline, 75 µm long, and 46 µm wide. Its inner edge is thickened.

Ctenidia are arranged merobranchially with the longest ctenidium on each side positioned the fourth from the posterior end. Size and number of ctenidia depend on animal size. The 8.7 mm long specimen has 16 ctenidia on the right and 17 on the left side; the juvenile (3.4 mm) has 7 ctenidia on each side of the foot (Fig. 2B).

Distribution

Known from the Cape of Good Hope (type locality), Shag Rocks near South Georgia Island and eastern Weddell Sea (this study) (Fig. 5). Bathymetrically the species lives between 284-1064 m depth (Thiele 1906b, Gutt *et al.* 2000, Sirenko and Schrödl 2001, Linse *et al.* 2006).

DISCUSSION

Since its description, *Stenosemus simplicissimus* was never recollected. Several expeditions to the Subantarctic Marion and Prince Edward Islands failed to locate this species, although the congeneric *Stenosemus exaratus* (G. O. Sars, 1878) [reported as *Stenosemus dorsuosus* (Haddon, 1886)] was found together with the following species: *Leptochiton kerguelensis* Haddon, 1886, *Hemiathrum setulosum* Carpenter in Dall, 1876, and *Placiphorella* sp. (Branch *et al.* 1991). *Leptochiton kerguelensis* and *H. setulosum* are typical faunistic elements of the Antarctica; the latter is *Placiphorella atlantica* (Verrill and S. I. Smith in Verrill, 1882) (pers. obs.).

Stenosemus simplicissimus was first rediscovered during the ANT XIII-3 (EASIZ I) – Antarctic expedition at St. 01: 71°03.10′S 11°25.50′W, at 462 m (Gutt *et al.* 2000). During subsequent expeditions, the species was found again but only in small numbers.

The Weddell Sea supports a high diversity of mollusc species and, not surprisingly, their numbers decrease with increasing depth, along with a significant decrease in total biomass (Brey and Gerdes 1997, 1998). During the ANDEEP III expedition (from the Cape Basin to Kapp Norvegia and across the Weddell Sea), 186 mollusc morphospecies (3801 specimens from 12 EBS and 19 AGT stations) were collected from depths ranging from 1000 to 4900 m (Linse *et al.* 2006).

In contrast to former expeditions in this area of comparable scope (Sirenko and Schrödl 2001), only two polyplacophoran specimens were found, the herein mentioned juvenile of *Stenosemus simplicissimus* and *Leptochiton kerguelensis* (ZSM Mol 20060001). The latter specimen was collected at station PS67/133-2 (62°46′44″S 53°02′34″W-62°46′20″S 53°04′08″W) at 1581-1582 m depth. This is the maximum depth reported for this species, which was for-

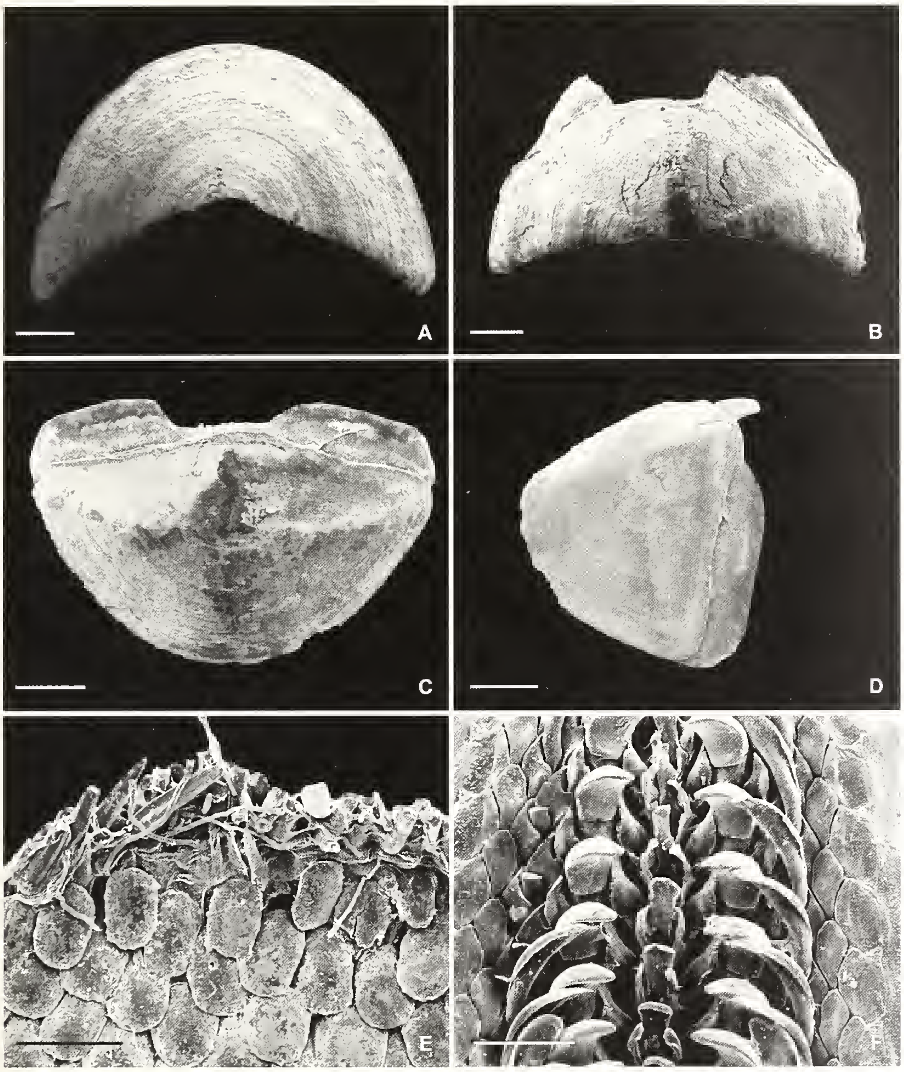

Figure 4. Scanning electron micrographs of *Stenosemus simplicissimus* (ZSM Mol 20008502), 8.7 × 4.2 mm. A, dorsal view of the head valve; B, dorsal view of valve ii; C, dorsal view of the tail valve; D, right lateral view of the tail valve, anterior at right; E, dorsal perinotum scales, close to the margin; F, rows 3-8 of the radula. Scale bars A-D: 500 μm, E-F: 100 μm.

merly only known from 1335 m in the Ross Sea (Dell 1990). A bulk of stones was found during the recent expedition, a substratum that allows settlement of chiton larvae. That they could be covered by sediment can be excluded because Paul (1976) has shown that chitons are able to remove sediment layers. Is the extremely low chiton diversity and density in the Antarctic merely a function of the great depth or related abiotic environmental conditions? Temperature is unlikely to be a problem as it does not change with depth (Brey and Gerdes 1998). The increase in water pressure can also be ruled out as diverse deep sea chiton faunas are known from other regions (Sirenko 2001). Most deep water chitons are dependent on plant remains (*e.g.*, sunken wood) that may not found in the Antarctic deep sea. The high organic food input (Brey and Gerdes 1997) in the Weddell Sea, together with abiotic conditions should favor colonization by non-herbivorous chitons. Several carnivorous taxa such as asteroids, ophiuroids, and polychaetes are highly adapted to the conditions of the Weddell Sea (Brey and Gerdes 1997, 1998). The chitons may lack the ability to co-occur with abundant predators such as the asteroids, which feed on chitons (Seiff 1975).

In summary, this report gives a detailed description of *Stenosemus simplicissimus*, contributes the first record of the species' juvenile stage, and extends its bathymetric range and that of *Leptochiton kerguelensis*, thus providing a further piece of the unfinished puzzle documenting Antarctic deep sea communities. The material obtained to date for *S. simplicissimus* suggests that it *could be* geographically restricted to the Southern Atlantic Ocean.

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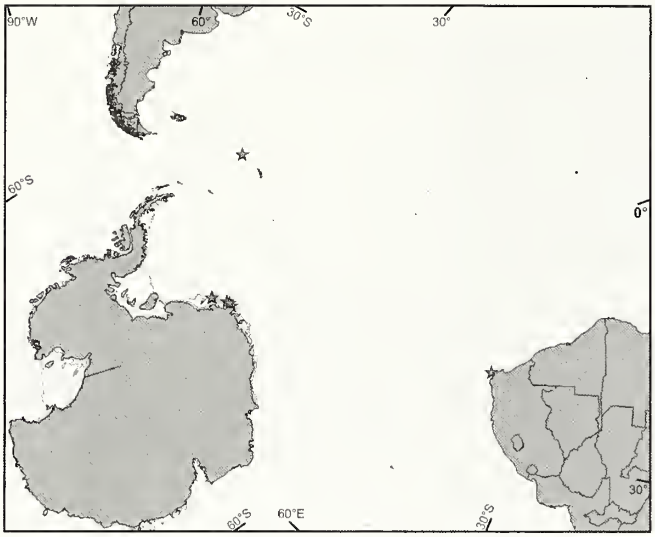

Figure 5. The geographical distribution of *Stenosemus simplicissimus.*

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Imposex level and penis malformation in *Hexaplex trunculus* from the Tunisian coast

Youssef Lahbib, Sami Abidli, and Najoua Trigui El Menif

Laboratory of Environment Bio-monitoring (LBE), Department of Biology, University of 7th November at Carthage, Faculty of Sciences of Bizerte, 7021 Zarzouna, Tunisia, lahbibyoussef@yahoo.fr and Youssef.Lahbib@fsb.rnu.tn

Abstract: *Hexaplex trunculus* (Linnaeus, 1758) is a gonochoric marine gastropod. Previous studies demonstrated that the biocide TBT (tributyltin) induced a sexual abnormality known as imposex (superimposition of male sexual characters onto females) in this whelk. Our study showed imposex in 19 stations out of 20 along the Tunisian coast. The frequency of imposex ranged from 0 to 100%. Among the 19 sites where the condition was found, 8 were considered as highly affected by imposex (VDSI > 3.7), 6 were moderately affected (VDSI > 1.3), and 4 were slightly affected (VDSI > 0). The most affected population was observed in the Bizerta Channel where the highest boating traffic was recorded; no imposex features were found in the Sea of Zarat where boating traffic was very low. Significant differences in imposex levels were obtained among sites with low, moderate, and high boating traffic. All the imposex indices values (I%, RPSI, RPLI, VDSI, FPL, and VDL) were significantly more elevated at sites with high boating traffic compared with sites with low and moderate boating traffic. Malformations of the penis were observed only in five stations and in very low rates, but where imposex rates were high. The incidence of penis malformation in males was significantly related to the boating traffic, I%, and VDSI. However, in females, a correlation was obtained only for the RPLI. The present study provides data on imposex level and penis malformations in *H. trunculus* from the Tunisian Coast that could be used as a starting point for future monitoring programs and for temporal trend surveillance related to TBT pollution in Tunisia where the use of TBT is not yet banned.

Key words: Muricidae, TBT-biomarker, marine pollution, Tunisia

Imposex (Smith 1971) or pseudo-hermaphroditism (Jenner 1979) is the development of male sexual characteristics (*i.e.*, a penis and/or a sperm-duct) in female prosobranch gastropods. The active biocide used in anti-fouling paints, TBT, was suspected to be the major cause of imposex since a direct correlation with shipping intensities was established (Bryan *et al.* 1986, Gibbs and Bryan 1986, Ten Hallers-Tjabbes *et al.* 1994, Axiak *et al.* 1995, Harino *et al.* 1998, Rilov *et al.* 2000, Fernandez *et al.* 2002). In contrast, even though there is little doubt that TBT has been the main cause of imposex in gastropods, it may be not the sole cause. Nias *et al.* (1993) reported that exposure to copper induces imposex. The same condition was also noted by Evans *et al.* (2000) in *Nucella lapillus* (Linnaeus, 1758) exposed to the estrogen-mimic nonylphenol. However, Davies *et al.* (1987) found imposex in 12% of the *N. lapillus* from a non-polluted site and considered it a natural phenomenon. Imposex has now been observed in about 150 gastropod species (Oehlmann *et al.* 2000).

In *Hexaplex trunculus,* observations on imposex were first reported along European coasts by Martoja and Bouquegneau (1988) in France, Axiak *et al.* (1995) in Malta, Terlizzi *et al.* (1998) in Italy, Vasconcelos *et al.* (2006) in Portugal, Rilov *et al.* (2000) in Israel, and Lahbib *et al.* (2004) in Tunisia. Some authors have indicated the development of malformations of the penis in males as well as in females of some gastropod species, namely *Hinia reticulata* (Linnaeus, 1758) (Stroben *et al.* 1992), *H. trunculus* (Terlizzi *et al.* 1998, Vasconcelos *et al.* 2006), and *Bolinus brandaris* (Linnaeus, 1758) (Ramon *et al.* 2001). However, descriptions of the penis malformations in *H. trunculus* were limited. The aim of the present work was (1) to provide data on imposex levels along the Tunisian Coast and (2) to describe malformations of the penis in *H. trunculus.*

MATERIALS AND METHODS

Adult *Hexaplex trunculus*, with shells of 40 to 60 mm in height and a sample size of 44 to 150 specimens per station, were collected between March and July 2004 from Tunisian coastal waters. Twenty locations were chosen according to the intensity of the marine traffic, from Bizerta to Djerba (Table 1, Fig. 1). For each zone, both the type of boating activity and the boat density were recorded. The number of working fishing boats in each area was obtained from annual statistical data of fishing activity in Tunisia in 2004 provided by the Ministry of Agriculture, or directly in some sites from fishermen. In sites with commercial activity, the number and type of boats were obtained from the annual report of the Tunisian commercial marine and harbors office in 2003.

Table 1. Collection data at the various stations. BT, boat type (F, fishing boat; P, passenger liner; M, merchant ship; O, oil tanker; F_b, ferry-boat); FD, fishing-boat density expressed in number of working boats in the area in 2004; CD, commercial boat traffic expressed in number of boats to/from the area in 2003; TC, traffic category (H, high; Md, moderate; L, low; *, site with low boating density but located nearby a commercial traffic line or harbor); M, male; F, female; *N*, number of individuals; SL, average shell length (mm); I%, imposex frequency; PL, average penis length (mm); RPSI, relative penis size index; RPLI, relative penis length index; VDSI, vas deferens sequence index; VDSr, vas deferens sequence range; VDL, mean vas deferens length (mm).

Site	BT	FD	CD	TC	Sex	*N*	SL	I %	PL	RPSI	RPLI	VDSI	VDSr	VDL
1. Bizerta Channel	FPMO	505	1014	H	M	63	41.7		12.05					
					F	37	46.5	100	8.23	33.03	69.13	4.24	4-5	12.17
2. Quarries Bay	F	120	—	H	M	26	41.9		11.81					
					F	37	49.2	100	7.77	28.47	65.79	4.09	4-4.3	12.29
3. Menzel Abderrahmen	F	150	—	H	M	33	44.9		14.64					
					F	29	41.7	100	5.18	5.13	37.16	3.73	3-4.7	12.12
4. Menzel Bourguiba	F	30	SA	H	M	29	52.9		12.25					
					F	27	55.7	100	3.54	2.69	29.96	3.97	3-4.7	12.20
5. Menzel Jemil	F	60	—	Md	M	20	44.1		13.56					
					F	40	43.1	66.6	0.28	0.00	2.05	1.31	0-4.3	3.53
6. El Azib	F	50	—	Md	M	29	43.9		14.33					
					F	21	46.2	62.0	0.37	0.00	2.52	1.28	0-3	2.64
7. Tunis North Lake	FPM	78	2444	H*	M	14	48.6		17.41					
					F	30	49	100	3.60	0.88	20.68	3.97	2-4.3	12.90
8. Small Gulf of Tunis	FPM	0	3001	H*	M	48	55.8		14.32					
					F	42	56.6	85.7	1.38	0.08	9.63	3.27	0-4.7	11.69
9. Khniss Lagoon	F	64	—	L	M	43	45.9		17.49					
					F	25	47.1	40	0.17	0.00	0.01	0.75	0-2	2.03
10. NPK Sfax	M	—	SA	H	M	51	42		10.31					
					F	30	44.1	100	5.63	16.98	55.38	4.00	4-4.3	12.91
11. Sfax Fishing Harbor	F	736	—	H	M	14	51.1		24.72					
					F	32	52.3	100	8.16	3.62	33.08	4.21	4-4.7	13.19
12. Gargour	F	30	—	H*	M	65	44.8		10.29					
					F	72	43.1	93.0	0.83	0.05	8.00	3.32	0-4	11.24
13. Skhira	FO	38	236	H	M	38	48.5		15.36					
					F	32	50.2	84.4	1.06	0.03	6.96	2.53	0-4	8.26
14. Gabes Fishing Harbor	F	108	—	H	M	46	52.5		14.78					
					F	33	54.2	51.5	1.60	0.02	5.48	1.82	0-4	5.91
15. Sea of Zarat	F	10	—	L	M	70	52.1		13.86					
					F	80	54.9	0.0	0.00	0.00	0.00	0.00	0	0.00
16. Adjim Channel	F_b	244	4	H	M	18	50.2		18.20					
					F	37	53.9	100	5.80	2.97	31.02	4.09	3-4.7	13.13
17. Gigthis-Djorf	F	20	—	L	M	62	42.9		9.16					
					F	81	46.6	6.2	0.02	0.00	0.09	0.13	0-3	1.70
18. Gigthis	F	52	—	Md	M	61	42.8		13.14					
					F	88	43	19.7	0.07	0.00	0.29	0.60	0-4	2.05
19. Guallala	F	10	—	L	M	26	42.9		14.28					
					F	27	46.6	3.7	0.00	0.00	0.05	0.04	0-1	0.10
20. Ain Meider	F	8	—	L	M	37	42.5		9.53					
					F	28	42.9	3.6	0.00	0.00	0.00	0.04	0-1	0.11

In the laboratory, the 1621 collected individuals were frozen. The shell was broken after thawing, and the soft tissues carefully removed. The mantles were longitudinally cut to reveal the pallial oviduct in females. Sexes were determined according to the presence or absence of the capsule gland and vagina. Normal females were separated from abnormal ones using a binocular dissecting microscope. In males and imposex-affected females, the length of the

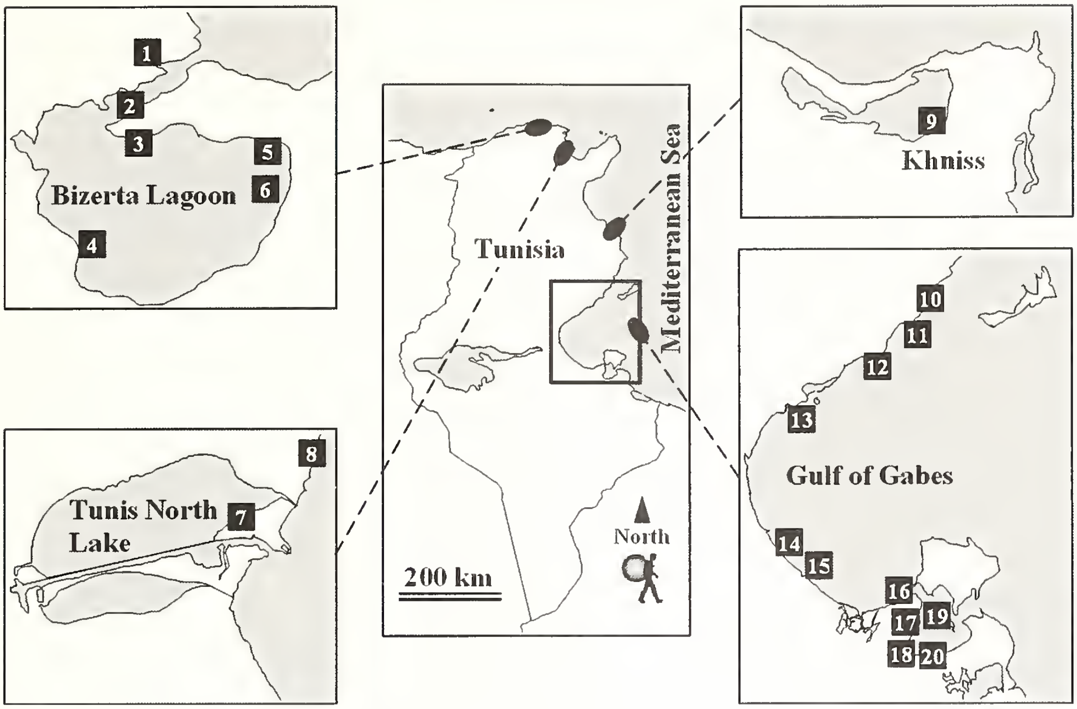

Figure 1. Sampling sites of *Hexaplex trunculus* along the Tunisian coast. 1, Bizerta Channel; 2, Quarries Bay; 3, Menzel Abderrahmen; 4, Menzel Bourguiba; 5, Menzel Jemil; 6, El Azib; 7, Tunis North Lake; 8, small gulf of Tunis; 9, lagoon of Khniss; 10, NPK Sfax; 11, fishing harbor of Sfax; 12, Gargour; 13, Skhira; 14, fishing harbor of Gabes; 15, Sea of Zarat; 16, Adjim Channel; 17, Djorf-Ghigthis; 18, Ghigthis; 19, Guallala; 20, Ain Meidder.

straightened penis (from the base of the penis to the end of the penial flagellum) and the vas deferens were measured using an ocular micrometer.

Imposex incidence and levels were quantified by using the following indices: (1) the imposex incidence or frequency (I% = percentage of imposex-affected females compared to the total number of females in the sample), (2) the relative penis size index [RPSI = (average length of female penises)^3 × 100/(average length of male penises)^3] according to Bryan *et al.* (1986), (3) the relative penis length index [RPLI = (average length of female penises × 100)/(average length of male penises)] according to Stewart *et al.* (1992), (4) the female penis average length (FPL), (5) the female vas deferens average length (VDL), and (6) the vas deferens sequence index [VDSI = (sum of imposex stage values of all females)/(total number of females)] following Gibbs *et al.* (1987). Some imposex stages and malformations affecting the penis were photographed under the binocular microscope using a digital camera.

For the statistical comparison of the data, each sampling station was assigned to 1 of 3 categories in terms of boating traffic density (Table 1): (1) high shipping density (>100 boats in the area per year), (2) moderate (50-100 boats in the area per year), and (3) low (<50 boats in the area per year). Sites with shipyard activities or located near a high traffic area were classified in the high category. All imposex indices (I%, RPSI, RPLI, VDSI, FPL, and VDL) were calculated for each category. The significance of differences in imposex levels between the 3 different categories of boating traffic were tested using one-way analysis of variance (ANOVA) and Chi-square test.

With regard to the malformation affecting the penis, the relationship with boating traffic and some imposex indices (I%, RPLI, and VDSI), recorded at sites where the condition was observed, was established, using a regression analysis.

RESULTS

Imposex distribution

Imposex was observed at 19 out of 20 sampling sites. However, the degree and the intensity of this alteration varied depending on the boating activity at each site (Table 1). The results indicated that the Bizerta Channel had the highest recorded imposex level with an RPLI of 69.08%, a VDSI of 4.23 and a sterility rate of 8%. High levels of imposex were also observed for Quarries Bay, Menzel Abderrahmen, Men-

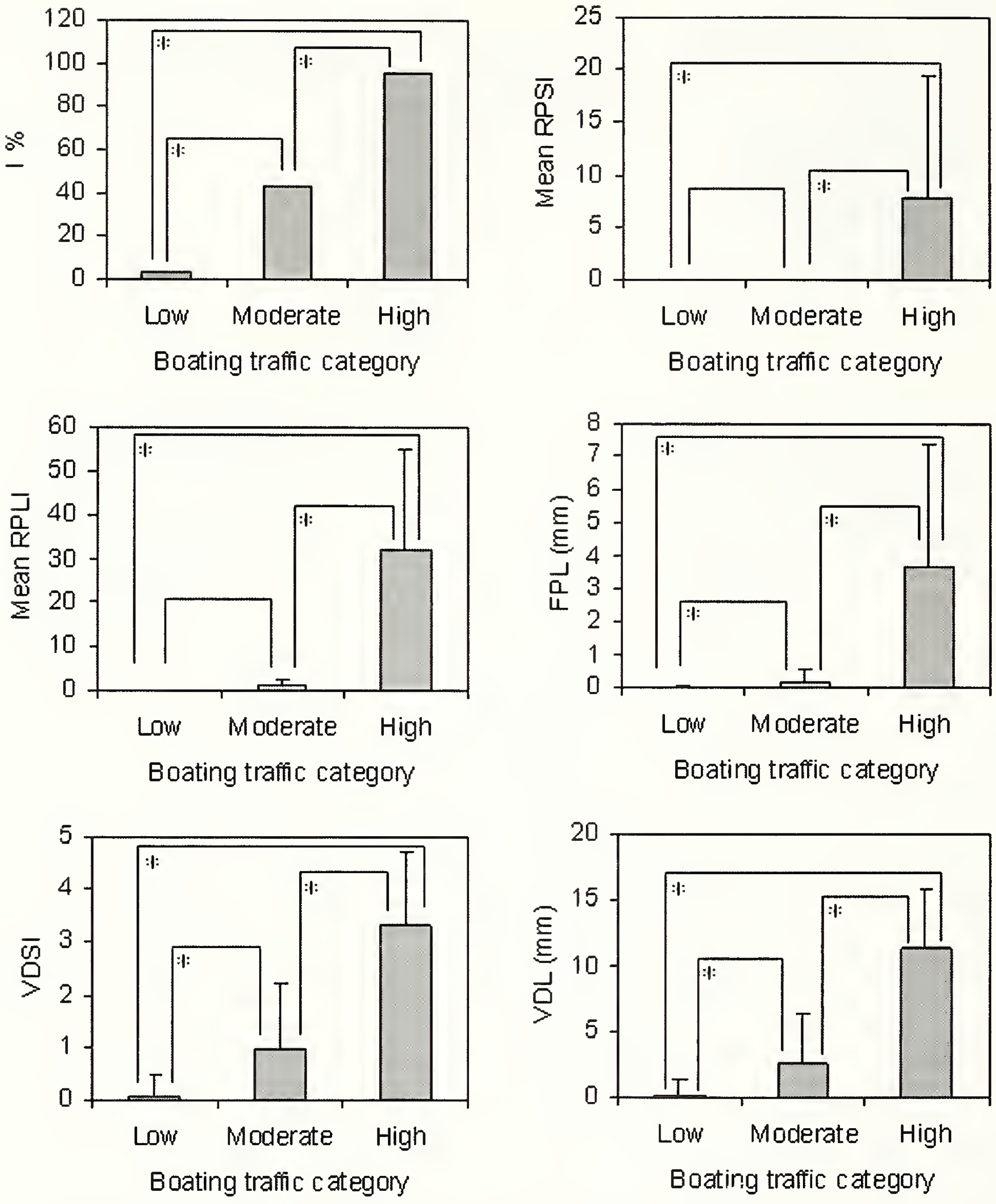

Figure 2. Imposex development in *Hexaplex trunculus* in relation to boating traffic. (*), significant difference ($P = 0.05$, one-way ANOVA applied to all indices except the I% index tested by a Chi-square test χ^2).

zel Bourguiba, Tunis North Lake, NPK Sfax, Fishing Harbor of Sfax, and Adjim Channel (VDSI above 3.7, Table 1). Moderate levels of imposex were recorded for Gargour, Skhira, fishing harbor of Gabes, Khniss, Small Gulf of Tunis, El Azib, and Menzel Jemil (VDSI >1.3, Table 1). At the rest of the stations (Guallala, Ain Meider, Gigthis-Djorf, and Gigthis), very low levels of imposex were recorded (VDSI above 0), while no female showing any form of genital disorder was observed at the Sea of Zarat (Table 1).

Significant differences were obtained between imposex indices and categories of boating traffic, indicating that imposex development is related to the intensity of marine traffic (Fig. 2). All the imposex indices values were significantly elevated in the high shipping traffic category of stations

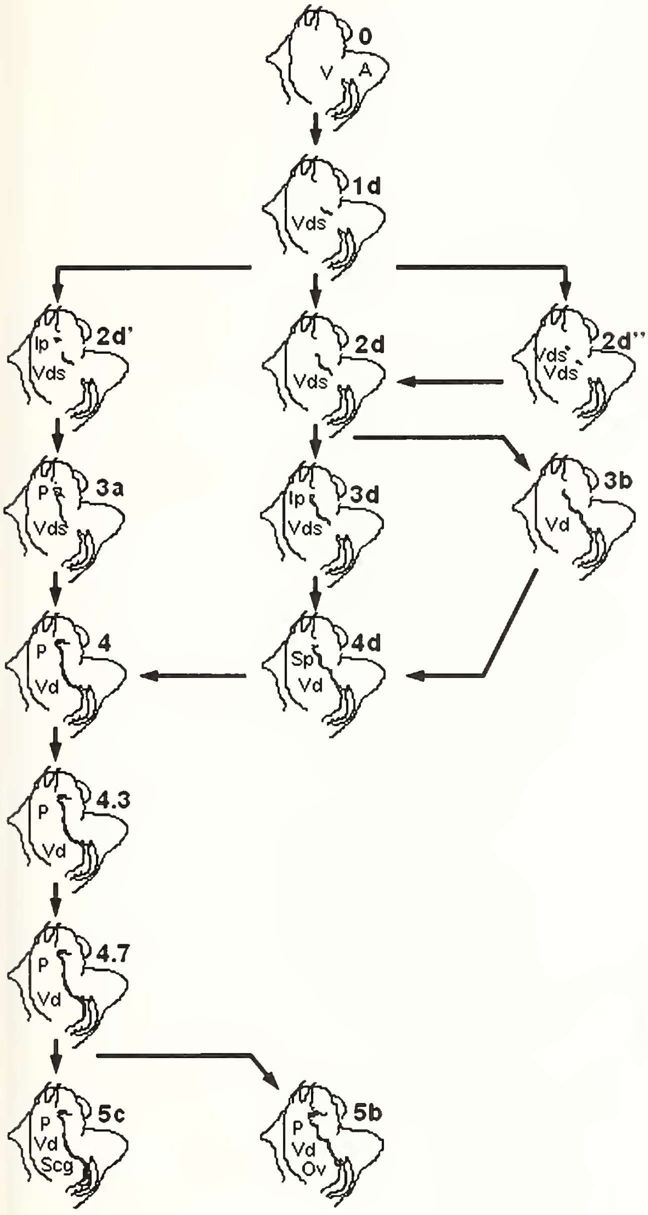

Figure 3. Schematic representation of the imposex pathways observed in *Hexaplex trunculus* from Tunisian waters. A, anus; Scg, split capsule gland; Ip, incipient penis; Ov, occluded vulva; p, penis; sp, small penis; V, vulva; Vd, vas deferens; Vds, vas deferens section.

compared to the moderate and low categories. Significant differences were also revealed between the moderate and the low categories for all imposex indices except RPSI and RPLI (Fig. 2).

Scheme of imposex pathways

The sequential stages of the imposex development observed in this study are illustrated (Fig. 3). The first imposex characters (1d, 2d, 2d’, and 2d”) were detected only at sites with low boating activity. Whereas some advanced stages (3b, 3d, 4d, 4, 4.3, and 4.7) were found at the more affected areas (Fig. 3). The more advanced stages of imposex recorded in this study were the VDS 5b and 5c. These stages, causing sterility in females, were only revealed in the Bizerta Channel.

In *Hexaplex trunculus*, females start to show evidence of imposex by the presence of a vas deferens section halfway between the penis site and the vagina (stage 1d). Thereafter, 3 cases are possible: (1) the vas deferens grows towards the proximal and distal sides (stage 2d), (2) an incipient penis develops behind the right ocular tentacle (stage 2d’), or (3) a second portion of the vas deferens appears (stage 2d”). At stage 3, females could show (1) a complete vas deferens without a penis (stage 3b), (2) an incipient penis linked to the vas deferens section (stage 3d), or (3) a small penis with penis-duct continuing in a portion of the vas deferens (stage 3a). At stage 4, the vas deferens reaches the vaginal opening, passes it (stage 4.3), and runs into the ventral portion of the capsule gland (stage 4.7). Sterility is reached in stage 5, in which the vulva is occluded by the development of the vas deferens tissue at the spawning aperture (stage 5b, Fig. 4A-B) or the capsule gland is split following the ventral development of the vas deferens (stage 5c, Fig. 4C).

Penis malformations in males

The normal male penis in *Hexaplex trunculus* is generally bent (Fig. 5A), it possesses a large base and a long fla-

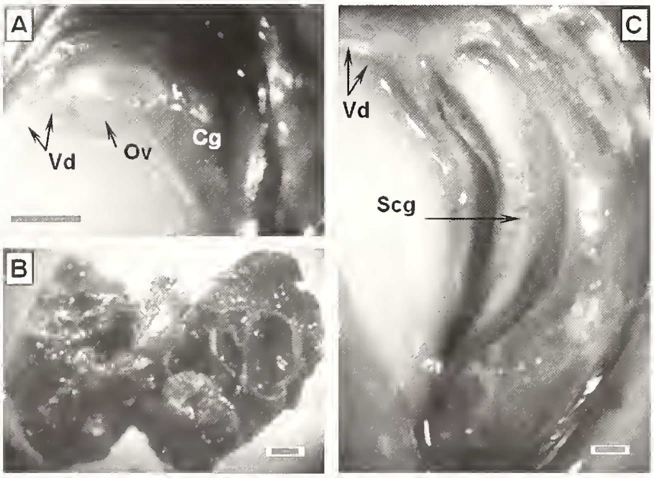

Figure 4. Imposex stage 5b (A, occluded vulva; B, dark mass of egg capsules removed from the capsule gland at this stage) and 5c (C) showing the split capsule gland. Cg, capsule gland; Scg, split capsule gland; Ov, occluded vulva; Vd, vas deferens (scale bar = 1 mm).

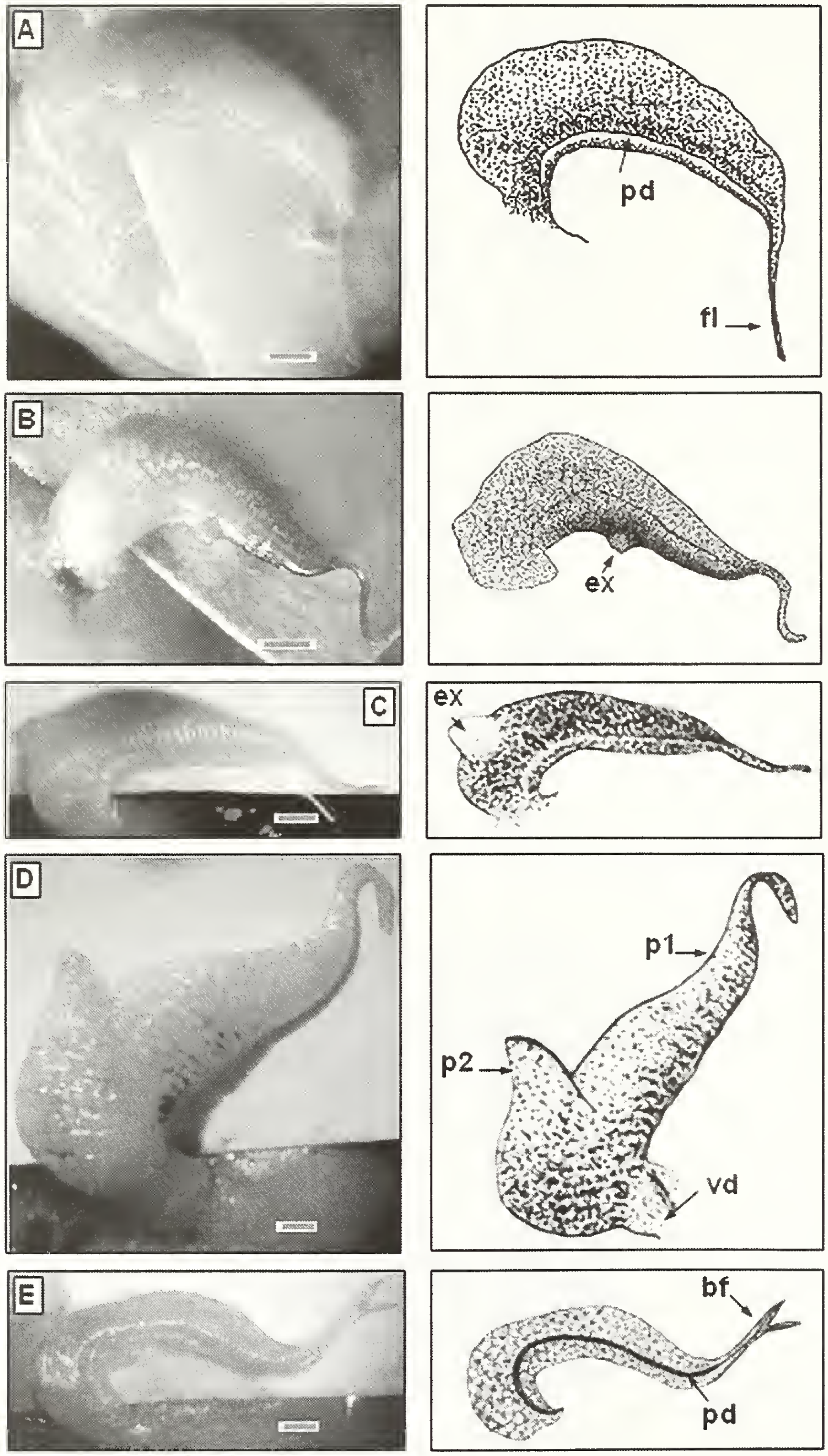

Figure 5. Penis malformations in male *Hexaplex trunculus*. A, normal penis. B-E, abnormal penises observed in Bizerta Channel (B), the small Gulf of Tunis (C-D) and the Tunis North Lake (E). bf, bifurcation; pd, penis duct; ex, excrescence; fl, flagellum; p1, penis 1; p2, penis 2; vd, vas deferens (scale bar = 1mm).

gellum-like tip reaching on fifth of the total penis length, depending on the reproductive season. The penis-duct is an open fissure, but it becomes a closed tube during reproductive activity. Male penis malformations were detected in the Bizerta Channel, Tunis North Lake, and the small gulf of Tunis with 1-2 affected individuals per station (Table 2). The malformation varied from the development of a tissue bud in both the anterior and the posterior sides of the penis (Fig. 5B-C) to the biphallic penis (Fig. 5D) and the bifurcated flagellum (Fig. 5E). A good correlation was obtained between the incidence of penis malformation, boating traffic, I%, and VDSI ($r > 0.85$, Fig. 6). However, the correlation with RPLI was moderate ($r = 0.49$).

Penis malformations in female

Malformations affecting the female penis were observed in 4 stations with a number varying from 1 to 3 affected individuals per station (Table 2). In Gabes and Bizerta Channel, malformations were characterized by the development of the bud tissue (excrescence) at the base of the penis (Fig. 7A) or half of the total penis length at the posterior side (near the penis-duct, Fig. 7B). Biphallic penis (Fig. 7C) and inflated penis tip (Fig. 7D) were revealed in Menzel Jemil and Tunis North Lake, respectively. The regression analysis showed a good relationship of the malformation rate only with RPLI. The correlations between boating traffic and I% and VDSI were relatively weak ($r < 0.34$, Fig. 6).

DISCUSSION

Imposex was found in most of the sites. High levels were recorded in sites frequently used by boats or located near a source of leaching TBT, such as harbors and shipyard activities stations. Moreover, the highest indices were recorded in areas where the predominant source of TBT involved large boats. However, the moderate level of imposex recorded in the fishing harbor of Gabes

Table 2. Genital malformation in *Hexaplex trunculus* from Tunisian coast with comparison to data collected from previous studies in the same snail and other species of gastropod. *N*, number of specimens; Na, number of affected individuals; VDS, vas deferens sequence; —, no data available.

			Males			Females			
Studies	Species	Locations	*N*	Malformation	Na	*N*	Malformation	Na	VDS
Present study	*Hexaplex trunculus*	Gabès Fishing Harbor	46	Absent	0	33	Penis excrescence	1	4
	Hexaplex trunculus	Steg Tunis North Lake	14	Bifurcated tip	1	30	Inflated penis tip	1	3
	Hexaplex trunculus	Small Gulf of Tunis	48	Double penis	1	42	Absent	0	—
				Penis excrescence	1				
	Hexaplex trunculus	Menzel Jemil	40	Absent	0	40	Double penis	1	2
	Hexaplex trunculus	Bizerte Channel	42	Penis excrescence	2	52	Penis excrescence	3	4.3, 4.7, and 5
Terlizzi *et al.* 1998	*Hexaplex trunculus*	Italian Coast	—	Bifurcated penis	—	—	Bifurcated penis	—	>4
				Branched vas deferens			Branched vas deferens	—	>4
Vasconcelos *et al.* 2006	*Hexaplex trunculus*	Ria Formosa (Portugal Coast)	621	Penis excrescence	2	562	Rounded penis tip	5	—
Ramon *et al.* 2001	*Bolinus brandaris*	Villanovai (Spain Coast)	59	Penis excrescence	1	75	Absent	0	—
Stroben *et al.* 1992	*Hinia reticulata*	Brittany and Normandy Coast	2760	Penis and/or vas deferens excrescences	4	3562	Bifurcated penis and double penis	10	4
							Coiled vas deferens	7	—

(51.5%), despite the high boating traffic recorded in the area (108 boats), is explained by the heterogeneity of the sampled population. At this station, many fishermen reject accidentally fished whelks found in fishing nets that come from locations far from the harbor where no imposex is present. According to imposex index values and the knowledge that TBT is the cause factor of imposex induction (Ten Hallers-Tjabbes *et al.* 1994, Mensink *et al.* 2002), the Bizerta Channel is considered as the most affected site along the Tunisian coast by TBT pollution, because sterility was recorded. The absence of imposex in the Sea of Zarat is explained by the weak intensity of boating traffic recorded at this site. The imposex level in *Hexaplex trunculus* in the present study, in comparison to other Mediterranean countries such as Italy (Terlizzi *et al.* 1998) and Malta (Axiak *et al.* 1995), suggests the Tunisian coast is relatively less polluted by TBT. Sterility was recorded in only one station among 20 with a frequency of 8%. Along the Italian coast, Terlizzi *et al.* (1998) reported the occurrence of sterility in 12 sites out of 15 with a frequency varying from 11.1% at Forio to 100% at SM di Pagana. In Malta, the most affected site had an RPSI of 98.1% and a VDSI of 4.8 (Axiak *et al.* 1995), compared to 33.03 and 4.23 in Tunisia. In the lagoon of Venice (Italy), Pellizatto *et al.* (2004) recorded a female penis length (FPL) of 12 mm, a RPSI of 36.02, and a VDSI of 4.9 in S. Nicolo del Lido, and 8, 8.03 and 4.1 in S. Maria del Mare. Compared with our study, FPL and VDSI indicated that imposex level is relatively similar in Quarries Bay (FPL = 7.77, VDSI = 4.09) and S. Maria del Mare. However, the difference observed in RPSI values (28.47 in Quarries Bay and 8.03 in S. Maria del Mare) is certainly related to the reproductive season in which males exhibit penis length variation. For this reason, we think that using the FPL to asses TBT pollution is more informative than using RPSI. According to Pellizatto *et al.* (2004), the level of TBT found in the entire organism of *H. trunculus* from S. Maria del Mare varied between 53 and 60 ng per g dry weight.

The morphological expression of imposex in Tunisian populations of *Hexaplex trunculus* was different at early stages from the general scheme of vas deferens sequence proposed for the same species in the Maltese Islands (Axiak *et al.* 1995) and Italian coast (Terlizzi *et al.* 1999). These authors mentioned that the first sign of imposex is the presence of an incipient penis behind the right ocular tentacle (stage 1a). Afterwards, the penis duct appeared (stage 2a) and the vas deferens developed progressively from the base of the penis toward the vaginal opening (stage 3a). In Tunisian waters, we observed that the first sign of imposex was expressed by development of a small portion of vas deferens halfway between the penis site and the vagina (stage 1d). Thereafter, the penis appeared following the d pathway (at stage 3d) or the d' pathway (at stage 2d'). These differences in imposex developmental stages observed between Tunisia and Italy and Malta could be explained by factors other than TBT. These factors could range from exposure to heavy metals (Nias *et al.* 1993), parasites (Gorbushin 1997), and other androgenic compounds (Cajaraville *et al.* 2000). Another hypothesis is genetic differences between Tunisian and European populations of *H. trunculus* that could also lead to distinct sequences of imposex induction. The development of a portion of the vas deferens as a first sign of imposex in Tunisian *H. trunculus* allowed calculating a new index, the VDL (mean length of the vas deferens), that gives more information on the level of imposex in the less affected area,

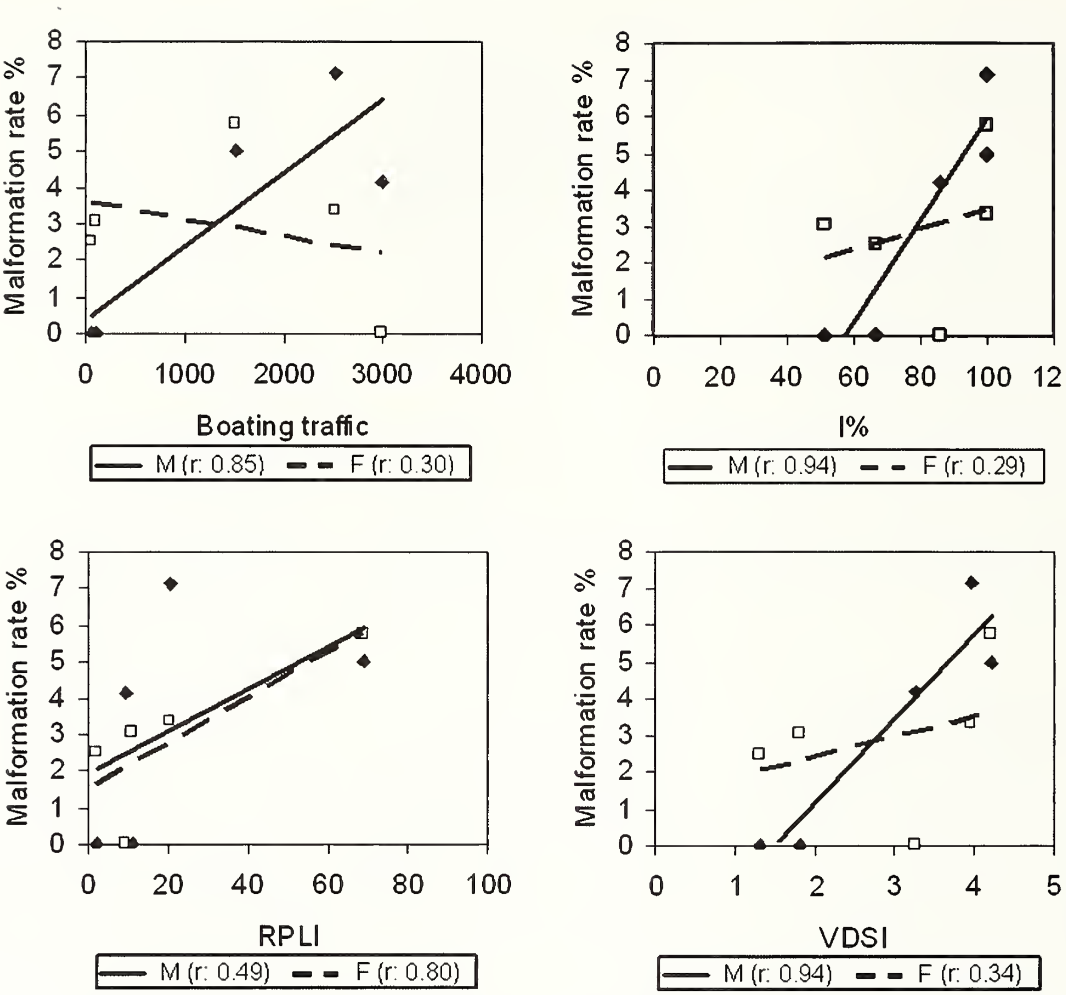

Figure 6. Regression analysis between penis malformation rate and boating traffic and some imposex indices (I%, RPLI, and VDSI) in both sexes. M, in males; F, in females; r, regression coefficient. Boating traffic was estimated as the number of existing boats and visiting boats in the area per year.

where penises in females are lacking or less developed, and RPSI and RPLI are close to 0. These indexes (RPSI and RPLI) are more informative in populations exhibiting the imposex (a) pathway (penis developed at first).

Penis morphological alterations in *Hexaplex trunculus* affected by imposex were reported by earlier authors, but no description nor pictures and schemes were provided. Terlizzi *et al.* (1998) observed penis bifurcation in *H. trunculus* in both sexes, but especially in males from the Italian coast. In the Ria Formosa lagoon (Portugal), Vasconcelos *et al.* (2006) found 2 males among 621 examined with penis excrescences and 5 females among 562 with a rounded penis tip. Compared to our study, the novelty was the observation for the first time of a penis with bifurcated flagellum in males and a penis with excrescence in females. No more than one excrescence per penis was observed in the present study, 1 in males and females, in comparison to male *Bolinus brandaris*, in which penises with many excrescences were found (Ramon *et al.* 2001). In male *Hinia reticulata* (Stroben *et al.* 1992) and in both sexes of *Nucella lapillus* and *Ocenebra erinacea* (Linnaeus, 1758) (Oehlmann *et al.* 1991, 1992), penis excrescences were also observed (Table 2). Stroben *et al.* (1992) have found females with bifurcated penises and females with two penises in *Hinia reticulata*. Contrary to the present study where no malformations associated to vas deferens development were revealed, Terlizzi *et al.* (1998) found branched vas deferens in *H. trunculus*, and Stroben *et al.* (1992) observed coiled vas deferens in *Hinia reticulata*.

With regard to the imposex stages in females in which the malformations were found, the abnormalities were observed since stage 2d', but especially in the more advanced stages. The correlation between malformed penises and the

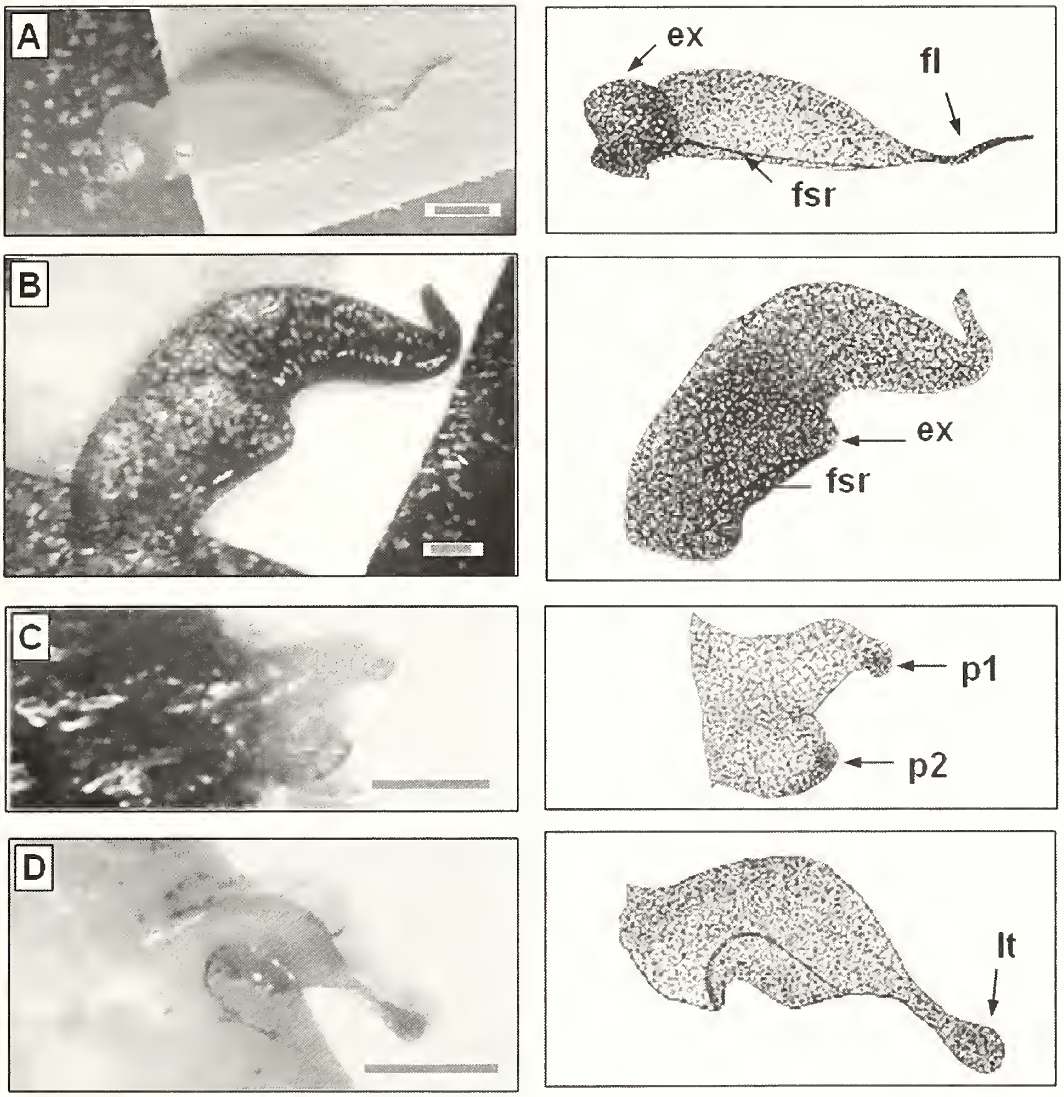

Figure 7. Penis malformations in female *Hexaplex trunculus*. A, in Gabes fishing harbor; B, in Bizerta Channel; C, in Menzel Jemil; D, in Tunis North lake; ex, excrescence; fl, flagellum; fsr, fissure of the vas deferens; lt, inflated tip; p1, penis 1; p2, penis 2 (scale bar = 1 mm).

boating traffic and imposex indices was significantly better in males than in females, except for the RPLI where the converse situation was recorded. These findings indicated that in males, the development of penis malformation is related to imposex level. However, in females, the occurrence of such conditions is related to the development of the penis and consequently to the imposex pathway (d or d'). The causal factor of penis malformation during development is unknown; it could be related to the presence of TBT in sea water, as reported by Mensink *et al.* (2002). These authors obtained in the laboratory penis malformations in juveniles of *Buccinum undatum* at 500 ng/L TBT. In our case, the development of such a condition in males is certainly linked to TBT pollution, because a good correlation with I% and VDSI was obtained. However, this hypothesis must still be supported by laboratory exposure of *H. trunculus* to TBT. Another hypothesis relates to the natural development of malformations, since the rate was low and relatively similar between males and imposex females. In this context, Garaventa *et al.* (2006) have reported the presence of biphallic males among museum specimens of *H. trunculus* collected before the use of TBT. Such data suggest the existence of natural abnormalities of the penis that are not related to TBT pollution.

This paper, reporting the imposex level and penis malformations in *Hexaplex trunculus* from the Tunisian coast, is important to assess the environmental consequences of the boating activity. The high values of VDSI in some populations suggest the existence of diffuse TBT pollution along the majority of the Tunisian coast. Further investigation of organotin in seawater, sediments, and gastropod tissue are in progress. Such data could be useful in regulation of TBT-based antifoulants and the legislation to ban the use of TBT-based paints in Tunisia.

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Threatened Bliss Rapids snail's susceptibility to desiccation: Potential impact from hydroelectric facilities

David C. Richards and Tristan D. Arrington

EcoAnalysts Inc., Center for Aquatic Studies, 11 E. Main Street, Suite M, Bozeman, Montana 59715, U.S.A., drichards@ecoanalysts.com and tarrington@ecoanalysts.com

Abstract: Water levels in the regulated Snake River, southern Idaho, U.S.A. can fluctuate daily and seasonally due to hydroelectric demands. The federally listed threatened Bliss Rapids snail, *Taylorconcha serpenticola* Hershler *et al.*, 1994 (Family: Hydrobiidae), survives in and near these fluctuation zones. Remaining *T. serpenticola* populations occur only in sections of the Snake River that are impacted by these hydroelectric facilities and associated springs. Because effects of rapid draw-down in fluctuation zones on *T. serpenticola* are unknown, we conducted a laboratory experiment to evaluate potential impacts of desiccation. Our experiment compared desiccation resistance at several air temperatures, on dry and wetted substrates, and for 'small' vs. 'large' snails. Probit regression-maximum likelihood models estimated lethal time (LT_{50}) values. Survival was significantly greater on wetted substrate than on dry substrate and was lowest at temperatures <0°C and at 37°C on dry substrate. Survival was greatest at 17°C on wetted substrate. There was no significant difference in survival at temperatures above 0°C on dry substrate other than at 37°C. LT_{50} survival ranged from 0.5 hours at −7°C to 157.0 hours at 17°C on wetted substrate. There were no significant differences in survival relative to snail size in any treatment. Our results suggest that desiccation could impact *T. serpenticola* populations if snails become stranded on dry substrates during rapid water-level fluctuations of the Snake River, particularly during subzero winter or extreme high summer temperatures. The most important factor determining survival would be the ability to find refuge on the undersides of cobbles, where snails typically occur, or in habitats that remained moist for the duration of the draw-down of the river.

Key words: regulated rivers, probit regression, population viability, threatened species, Snake River

The federally listed, threatened Bliss Rapids snail *Taylorconcha serpenticola* Hershler *et al.*, 1994 (Family: Hydrobiidae) (Fig. 1) occurs only in fragmented populations within approx. 80 river kilometers of un-impounded sections of the regulated Snake River and in associated cool to cold-water springs of the Snake River aquifer, south-central Idaho, U.S.A. (Upper Snake River Basin) (Hershler *et al.* 1994, Richards 2004, Richards *et al.* 2006) (Fig. 2). Water levels in the un-impounded sections of the river fluctuate daily and seasonally depending on flows, location, geomorphology, and weather conditions. Daily fluctuations, mostly a result of hydroelectric generation from three dams in this area (Fig. 2), occur for only several hours at a time (Stephenson *et al.* 2004). Populations of *T. serpenticola* occur within fluctuation zones and may be affected by daily fluctuations, whereas spring populations are not subjected to these same fluctuations. Direct effects of rapid dewatering on individual *T. serpenticola* survival are unknown.

Taxonomic history and status of *Taylorconcha serpenticola*

Taylorconcha serpenticola was first collected in the Snake River of south-central Idaho and recognized as a new taxon by Taylor in 1959 (Taylor 1982). The taxon, although apparently collected and noted as early as 1884, went undescribed until Hershler *et al.* (1994) placed the snail in the new genus *Taylorconcha* and a new species, *Taylorconcha serpenticola*. Hershler *et al.* (1994) described the known distribution of this species as the main stem Snake River and associated springs of south-central Idaho.

The origins of *Taylorconcha serpenticola* are distinct in the molluscan fauna. *Taylorconcha* can be traced back to the late Pliocene (Blancan) Glenns Ferry formation in Gooding County, Idaho; the early Pleistocene Bruneau formation in Owyhee County, Idaho; and the late Pleistocene and probable Holocene deposits in Gooding County (Smith *et al.* 1982, Hershler *et al.* 1994). Of equal significance, *Taylorconcha* can be identified as a survivor of the Pliocene Lake Idaho, geologically dated about 3.5 Ma (Hershler *et al.* 2006).

Taylorconcha is one of the few remaining extant taxa from ancient Lake Idaho, which once supported a molluscan fauna of more than 80 endemic taxa (Hershler *et al.* 1994). Lake Idaho was thought to have extended from the border between western Idaho and eastern Oregon upstream of Hells Canyon eastward to a point near American Falls, Idaho (Taylor 1985, Hershler *et al.* 1994). Remnant populations of *T. serpenticola* remain in Idaho, inhabiting approx. an 80-km stretch of the Snake River upstream and downstream of Hagerman, Idaho in the Thousands Springs reach of the Snake River. *Taylorconcha serpenticola* was known historically from the main stem middle Snake River and associated

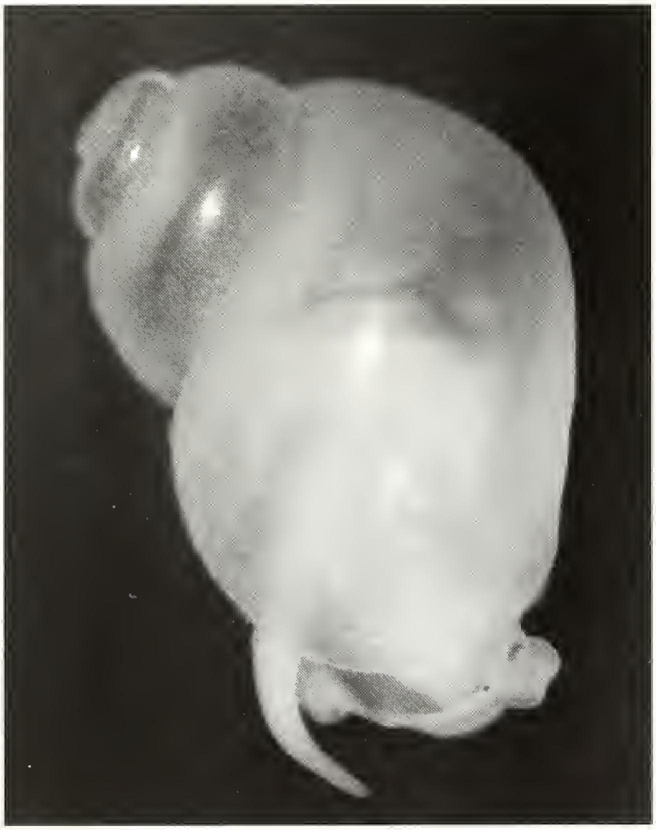

Figure 1. Male *Taylorconcha serpenticola.* Photo courtesy of Dan Gustafson, Montana State University, and David Richards, Eco-Analysts Inc., Center for Aquatic Studies, Bozeman, Montana.

springs between Indian Cove Bridge (Rkm 845.6) and Twin Falls (Rkm 982.5) (Hershler *et al.* 1994). Taylor (1982) believed that prior to dam construction there was probably a single population throughout this range.

The status of extant *Taylorconcha serpenticola* populations is a topic of concern. Federal action began on the species primarily in response to petitions submitted in 1980, under section 4(b)(3) of the Endangered Species Act (ESA) 1973. The snail was a candidate for category 1 listing from 1984 through December 18, 1990. This 1990 proposed rule listed *T. serpenticola* as an endangered species along with four other aquatic snails: the Snake River physa *Physa natricina* (Taylor, 1988), the Idaho springsnail *Pyrgulopsis idahoensis* (now Jackson Lake springsnail *Pyrgulopsis robusta* (Walker, 1908) (see Hershler and Liu 2004), the Utah valvata *Valvata utahensis* (Call, 1884), and the Banbury Springs *Lanx* (Frest, 1988) (limpet). On December 14, 1992, the U.S. Fish and Wildlife Service classified *T. serpenticola* as threatened while still taxonomically an "undescribed Hydrobiid" (U.S. Fish and Wildlife Service 1992).

Potential susceptibility of *Taylorconcha serpenticola* to desiccation

Taylorconcha serpenticola is the smallest (2.0-4.0 mm) of the Snake River hydrobiids in south-central, Idaho, and of those that we have observed, it is also the slowest moving (Richards 2004). Field trials indicated that *T. serpenticola* could travel approx. 1 to 10 cm/hour in water, which was more than ten times slower than the common pebble snail, *Fluminicola* (Carpenter 1864), and up to 100 times slower than the invasive New Zealand mudsnail *Potamopyrgus antipodarum* (Gray, 1843) (Richards and Arrington, unpubl. data). Thus, *T. serpenticola* is perhaps the least able of the hydrobiid species to actively avoid desiccation in fluctuation zones in the Snake River.

Life history and temporal environmental conditions may also affect *Taylorconcha serpenticola*, as has been shown for *Potamopyrgus antipodarum* survival probability to desiccation (Richards *et al.* 2004). Their results showed that: (1) smaller size classes had lower survival than larger size classes; (2) higher temperatures were related to decreased survivability; (3) freezing rapidly decreased survival; and (4) survival was greater at higher than lower humidity.

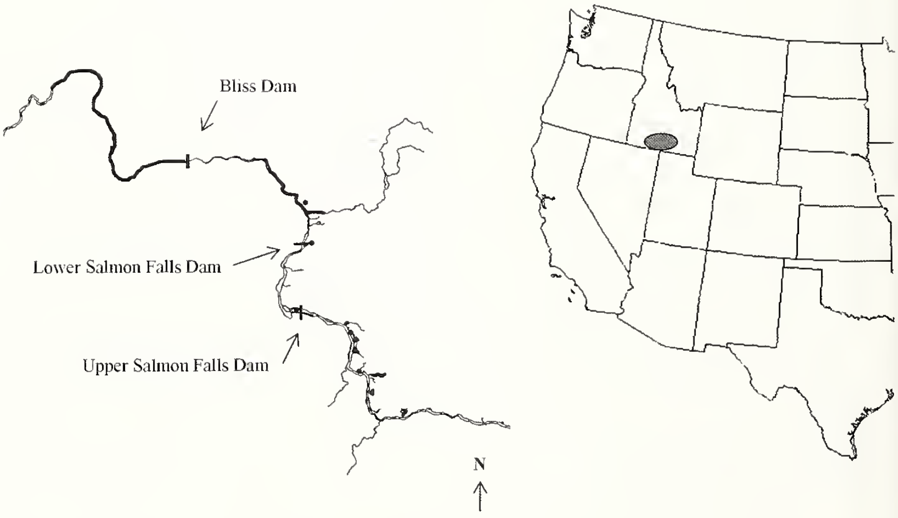

Figure 2. Current known distribution (approx. 80 river kilometers) of *Taylorconcha serpenticola* in the upper Snake River basin, south-central Idaho, U.S.A. Dark lines and dots indicate current known locations of the species.

Although *Taylorconcha serpenticola* is potentially susceptible to high rates of desiccation-induced mortality, this species, like all hydrobiid snails, has an operculum, which may help it survive. Winterbourn (1970) reported that *Potamopyrgus antipodarum* from New Zealand was able to survive desiccation for up to 50 days on a wetted substratum at 20-25°C. Richards *et al.* (2004) showed that *P. antipodarum*, in the western U.S.A., a parthenogenic clone, can survive desiccation on wetted substratum at 9°C for at least 48 hours.

The purpose of this study was to evaluate the survival probability of different sizes of *Taylorconcha serpenticola* under different time periods of desiccation at various temperatures and substrates (dry and wetted). Based on results of effects of desiccation on *Potamopyrgus antipodarum* (Richards *et al.* 2004), we hypothesized that *T. serpenticola* survival probability to desiccation was: (1) positively correlated with snail size, (2) negatively affected by increased temperature, (3) greater on wetted substrate than on dry substrate, and (4) negatively affected by freezing.

MATERIALS AND METHODS

Raising and rearing procedures

Taylorconcha serpenticola used in our experiments were from brood stock (250 individuals) collected in 1999 at the outlet of Banbury Springs, near Hagerman, Idaho. The *T. serpenticola* population at the outlet of Banbury Springs had the highest densities reported for any *T. serpenticola* population (>3000/m^2 in summer)(Richards 2004) and was considered the least likely population to be affected by 'harvest' for our experiments. Brood stock was supplemented with 100-200 individuals once to twice per year from the same source, until 2005. The collected snails and offspring were reared at EcoAnalysts Inc. Research Laboratory, Bozeman, Montana under the authority of a U.S. Fish and Wildlife Service Section 10 permit. *Taylorconcha serpenticola* populations in the lab were maintained in twelve to sixteen, 37.85-L aquaria at 16-17°C. Varying light: dark regimes were used to simulate or accelerate natural light conditions and seasons in an effort to produce more snails. All aquaria had substantial aeration, moderate flow, and contained various substrates, including periphyton-covered cobbles (food resource) that were collected from the outlet of Banbury Springs and the Snake River. Aquaria also contained native aquatic macrophytes including *Myriophyllum* sp., *Ceratophylum* sp., and *Elodea* sp. *Taylorconcha serpenticola* reproduced slowly in the laboratory (<5-7 eggs/year) (Richards and Arrington, unpubl. data); therefore, the number of individuals available for potential experimental sacrifice restricted experimental designs.

Experimental design

Twenty 'small' (1.50-2.00 mm shell height) and twenty 'large' (2.01-2.50 mm) *Taylorconcha serpenticola* were exposed to six air temperatures (−7, 0, 7, 17, 27, and 37°C) on two substrate conditions (dry and wetted) and ten time periods (2, 4, 8, 16, 24, 48, 72, 96, 120, and 144 hours for 0, 7, 17, and 27°C; 0.5, 1, 2, 4, 8, 16, 24, 48, 72, and 96 hours at −7 and 37°C). At −7°C only one substrate was used (dry) and at 7°C only one size class was used (1.5-2.50 mm). A total of 4360 snails were assayed in this experiment. The experiment was conducted in September 2004. The six temperatures were selected based on conditions likely to be encountered by *T. serpenticola* in the Snake River throughout the year. Based on past experiments (Richards 2004), a water temperature of 17°C appears to be the best temperature to promote *T. serpenticola* growth. The two substrate treatments were chosen to simulate conditions that *T. serpenticola* would encounter either on the tops of cobbles (dry) or the bottoms of cobbles (wetted) during dewatering.

Twenty 'small' and twenty 'large' *Taylorconcha serpenticola* were uniformly distributed on either a dry paper towel or wetted paper towel within a large, covered Petri dish. To reduce the effect of run order, the sequence of temperature treatments was randomized. Dishes were then placed in an environmental chamber and held at the appropriate temperature. Snails were removed from the chamber after the appropriate treatment interval and were transferred into new dishes filled with aquaria water at 15 to 17°C. After one hour in the water-filled dish, snails were observed to see if they opened their opercula and started crawling or if they were attached to the dish. If snails were crawling or attached to the dish, they were classified as 'alive'. If snails were not moving or not attached to the dish, they were observed under a dissection scope at 40× magnification; if no movement was apparent, snails were classified as 'dead'. Snails classified as 'dead' were then re-observed after 24 hours in water-filled Petri dishes. If snails were crawling or attached to dishes they were classified as 'alive'; if there was no observed movement, they were classified as 'dead'. Mortalities were kept as voucher specimens at EcoAnalysts Inc. Research Laboratory, Bozeman, Montana.

As a control, 20 'small' and 20 'large' *Taylorconcha serpenticola* were placed into two separate Petri dishes that were filled with aerated aquaria water for 120 hours. Controls were maintained at ambient lab temperature (16-17°C) and replenished with aquaria water as needed.

Probit regression was used to develop distribution models of survival for both 'small' and 'large' snails at the six temperatures. This method is widely used to evaluate dose/response of pesticides on insects and in medical studies (Finney 1971, Preisler 1988, Preisler and Robertson 1989, Baker *et al.* 1995, Peng *et al.* 2002). Compared with an

ANOVA, ANOVAs only determine if there is a significant difference between treatments, but probit analysis determines significant differences between treatments at any desired percent survival level. Probit analysis also models the relationship between percent survival and duration of exposure at any given temperature.

The most appropriate probit regression distribution model (Weibull, normal, lognormal, logistic, etc.) with 95% confidence intervals (CIs) was selected using maximum likelihood methods. Pearson chi-square goodness-of-fit tests were used for evaluating and selecting models. Goodness-of-fit tests were also used for comparing slopes of models. In addition, probabilities of survival, including LT_{50} values, were calculated; these are commonly used metrics for invertebrate bioassays (Dunkel and Richards 1998). A LT_{50} is the lethal time (or temperature) at which 50% of individuals being tested have died.

Treatment effects were considered significant if there was no overlap in 95% CIs of the probit models. All analyses were conducted using MINITAB 14.1 (Minitab Inc. 2003) and S-PLUS 6.1 (Insightful Corp. 2002).

RESULTS

Probit survival distributions were highly variable between treatments and significantly greater on wetted substrate than on dry substrate for both 'small' and 'large' snails

Table 1. Probit regression models and LT_{50} values of *Taylorconcha serpenticola* survival probability to desiccation, using best-fit maximum likelihood estimates. NA, not applicable.

Temp.	Best-fit probit regression model distribution	Pearson χ^2 goodness-of-fit test (*df*, *P*-value)	χ^2 test for equal slopes (*df*, *P*-value)	Log-likelihood	LT_{50} hr (95% CI)	
					Small	Large
−7°C	Weibull	1.68 (17, 1.00)	0.11 (1, 0.74)	−47.49	0.47 (0.34, 0.60) Dry 6.82 (2.53, 8.66)	0.47 (0.33, 0.60) Dry 8.75 (6.88, 11.08)
0°C	Lognormal	30.21 (35, 0.70)	8.39 (3, 0.04)	−221.78	Wetted 28.90 (23.15, 35.85)	Wetted 26.98 (21.64, 33.41)
7°C[a] (Dry)	Normal	0.07 (7, 1.00)	NA	−31.55	4.20[a] (2.45, 5.42)	
7°C[a] (Wetted)	Lognormal	7.83 (9, 0.55)	NA	−102.15	73.33[a] (57.04, 108.37)	
17°C (Dry)	Weibull	5.68 (17, 0.99)	2.23 (1, 0.14)	−69.49	2.33 (1.87, 2.97)	3.14 (2.61, 3.88)
17°C (Wetted)	Logistic	25.25 (17, 0.09)	0.09 (1, 0.76)	−100.07	131.96 (115.93, 154.09)	156.56 (140.58, 181.08)
27°C (Dry)	Weibull	5.38 (17, 0.99)	1.28 (3, 0.26)	−80.98	2.26 (1.82, 2.84)	3.22 (2.57, 4.07)
27°C (Wetted)	Logistic	13.50 (17, 0.70)	0.01 (3, 0.94)	−178.32	89.87 (75.87, 106.36)	88.59 (74.70, 105.91)
37°C	Lognormal	28.90 (35, 0.76)	5.21 (3, 0.16)	−289.41	Dry 0.98 (0.64, 1.47) Wetted 3.08 (2.16, 4.34)	Dry 1.52 (1.02, 2.21) Wetted 3.38 (2.38, 4.73)

[a] At 7°C only one size class was used (1.5-2.50 mm) due to limited number of snails available.

within each temperature treatment (Table 1). Survival was slightly less for 'small' snails than 'large' snails within each temperature treatment at most temperatures on both dry and wetted substrates but was not statistically significant (Table 1). Survival consistently decreased with increased desiccation times. Survival was significantly lower at −7°C than for any other temperature and almost identical for 'large' and 'small' snails (Table 1). Snails on wetted substrate survived >15 times longer than snails on dry substrate at 7°C (Table 1), and snails on wetted substrate survived >50 times longer than snails on dry substrate at 17°C (Table 1). There were no mortalities for 'small' snails ($N = 20$) and a single mortality (5%) for 'large' snails ($N = 20$) in the controls. Therefore, survivability was considered to be a result of treatment effects (*i.e.*, desiccation).

DISCUSSION

Our experiments showed that temperature and substrate moisture level could affect survival of *Taylorconcha serpenticola* to desiccation under controlled conditions. Our experiments simulated conditions that would occur on the tops (dry) and bottoms (wetted) of cobbles during flow fluctuations in the Mid-Snake River. Given that *T. serpenticola* moves relatively slowly (Richards 2004), its ability to avoid rapidly retreating water levels is limited. Therefore, the most important factor for individual survival is snail location at the time of receding water levels. Survival may be dictated by whether snails: (1) become stranded on the tops or sides of cobbles, where desiccation to temperature extremes is more likely, or (2) find refuge on the undersides of cobbles or in habitat that remains moist for the duration of the drawdown of the river.

Richards (2004) documented the snail's preference for sides or undersides of cobbles at the outlet of Banbury Springs and that it was only occasionally found on tops of cobbles. Bowler (2001) reported nocturnal movement of *Taylorconcha serpenticola* from the bottom to tops of cobbles. This preference for undersides of cobbles or 'photophobic tendency' may benefit *T. serpenticola* during rapid dewatering of shoreline habitat. Richards *et al.* (2005) and Stephenson *et al.* (2004) reported that densities of *Taylorconcha* are often greatest at shallow depths near the shoreline. Because these habitats are the most intensely affected by fluctuating river levels, a higher proportion of the river populations may be subjected to desiccation. Due to different shoreline topographies, it is impossible to determine fluctuation levels at any one location in the river and therefore, how much *T. serpenticola* habitat or what percentage of the population is affected at any point in time.

No research has been conducted on which stages of its life cycle are the most critical to population viability. For example, egg survival may be less important to population viability than survival of larger, more fecund adults or small to medium-sized snails that may have greater lifetime reproductive potential (Beissinger and McCullough 2002). It is also unknown if there is a seasonal effect of exposure on *Taylorconcha serpenticola* survival due to density-dependent interference or exploitative intraspecific competition. There is ample evidence for density-dependent regulation occurring in river invertebrate populations, including snails (McAuliffe 1984, Hart 1985, 1987, Lamberti *et al.* 1987, Osenberg 1989, Peckarsky and Cowan 1991, Kohler 1992, Anholt 1995) and possibly *T. serpenticola* (Richards and Arrington, unpubl. data). Exposure may reduce intraspecific competition of *T. serpenticola* by reducing densities, but may increase interspecific competition. For example, *Potamopyrgus antipodarum* and *T. serpenticola* are often found together in the Snake River fluctuation zones and have been shown to compete for limited food resources under *in situ* experiments (Richards 2004). Because *P. antipodarum* is better able to actively avoid exposure than *T. serpenticola*, flow-fluctuations may favor *P. antipodarum* over *T. serpenticola*, thus giving the former species an additional competitive advantage. Other biotic factors that were not evaluated, such as increased predation or parasite load, may also affect *T. serpenticola* survival during exposure.

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Field observations of the nocturnal mantle-flap lure of *Lampsilis teres*

Andrew Lee Rypel

Department of Biological Sciences, The University of Alabama, Box 870206, Tuscaloosa, Alabama 35487-0206, U.S.A., andrewrypel@yahoo.com

Abstract: Three yellow sandshell mussels, *Lampsilis teres* (Rafinesque, 1820), were observed in Lake Tuscaloosa, Alabama, and the temporal display pattern of their mantle-flap lures was investigated *in situ*. All three gravid females fully displayed their mantle-flap lures after dark during each nighttime visit ($N = 3$) but none displayed their lures during daytime ($N = 3$). An encounter between a mantle-lure and a largemouth bass was observed. These observations are the first reported of *in situ* mantle-flap lure displays and fish host encounters for *L. teres*, and support previous studies of diel display patterns in other mantle-lure displaying mussels. This diel lure display may be related to the ecology of the fish hosts they seek to attract. Future daytime and, especially, nighttime field observations of bivalve mussels with mantle-flap lures may greatly improve understanding of their reproductive ecology.

Key words: Bivalvia, diel, mussel, unionid, largemouth bass

Gravid, mature females of the mussel genus *Lampsilis* Rafinesque, 1820 display elaborate mantle-flap lures to attract fish hosts for glochidial larvae (Ortmann 1914, Kraemer 1970, Haag *et al.* 1999). Mantle-flap lure displays vary in response to time of day, light conditions, and presence of suitable fish hosts, and there appear to be interspecific differences in when displays begin (Kraemer 1970, Haag and Warren 2000). These variations may be related to the diel habits of the fish hosts used by each mussel species (Welsh 1933).

Lampsilis teres Rafinesque, 1820 is a unionid bivalve with a wide distribution from Mexico north to Minnesota and is found in the Mississippi, Rio Grande, Mobile, and Gulf drainages (Parmalee and Bogan 1998). The species is especially common in the southeastern U.S.A., prefers pool and shallow sandbar habitats (Ortmann 1926, Parmalee and Bogan 1998), and is often abundant in river impoundments (C. Lydeard, Smithsonian Institution, pers. comm.). Known fish hosts of *L. teres* include alligator gar (*Atractosteus spatula*), black crappie (*Pomoxis nigromaculatus*), white crappie (*Pomoxis annularis*), green sunfish (*Lepomis cyanellus*), largemouth bass (*Micropterus salmoides*), longnose gar (*Lepisosteus osseus*), orangespotted sunfish (*Lepomis humilis*), shortnose gar (*Lepisosteus platostomus*), shovelnose sturgeon (*Scaphirhynchus platorynchus*), and warmouth (*Lepomis gulosus*) (Watters 1994).

Previous studies (Kraemer 1970, Trdan 1981, Haag and Warren 1999, Haag and Warren 2000) have documented the reproductive strategy of displaying mussels and showed, under laboratory conditions, that mantle-lure displays respond to presence of fish hosts, light conditions, and substrate disturbance. However, no studies have reported *in situ* field observations of mantle-flap lure displays, and I briefly describe field observations of the mantle-flap lure of *Lampsilis teres*, including morphology, display timing, and physical encounters with fish.

MATERIALS AND METHODS

In May 2005 in Lake Tuscaloosa (an impoundment of the North River, Mobile River basin), Tuscaloosa County, Alabama, three individuals of *Lampsilis teres* (yellow sandshell) were observed displaying mantle-lures after dark beneath a boat dock (depth *ca.* 1.4-2.0 m). Using a flashlight, I observed each specimen from the dock, presumably without affecting the display pattern of their mantle-lures. Subsequent visits to this site were made over the next three days (11-13 May 2005, 12:00-4:00 PM) and three nights (11-14 May 2005, 9:00 PM-1:00 AM) to observe diel display behavior. Observations on the timing, morphological characteristics of the lure, and any interactions with fish were noted.

RESULTS

Display timing

All three specimens were in full display during each nocturnal visit, and no lures were displayed during daylight visits. The mussels were buried in the sediment at a slight angle (posterior facing up) with their mantle-flap lures fully extended and pulsating. During one visit just prior to dusk, none of the mussels were displaying; however, as the sun set, one specimen began to display. After sunset, the other two mussels began displaying their lures. During this twilight period, one mussel slowly moved from a horizontal position adjacent to a rock to soft sediment where it positioned itself vertically and began to display. The displays were periodic and occurred in episodes of various lengths. Total palpitations per episode of all 3 mussels ranged from 4 to 26 before individuals rested. Palpitating episodes lasted for 6 to 177 s and rest periods ranged in time from 10 to 98 s before recommencement.

Lure morphology

Mantle-lures were approx. 3-4 cm maximum length and 2 cm in maximum width (Fig. 1). The tissue was a dark pink color in the interior and white and tan on the margins. When fully displayed, the margins were wavy in appearance and resembled small fishes. The lures varied slightly in morphology and color depending on the individual, but the margin of all lures undulated during displays, while the interior of the lures pulsated. As the display was initiated, the lure would extend slowly from the mantle, motionless at first, and slowly begin to palpitate. When only moderately or minimally displayed, the lure appeared to have less motion and color.

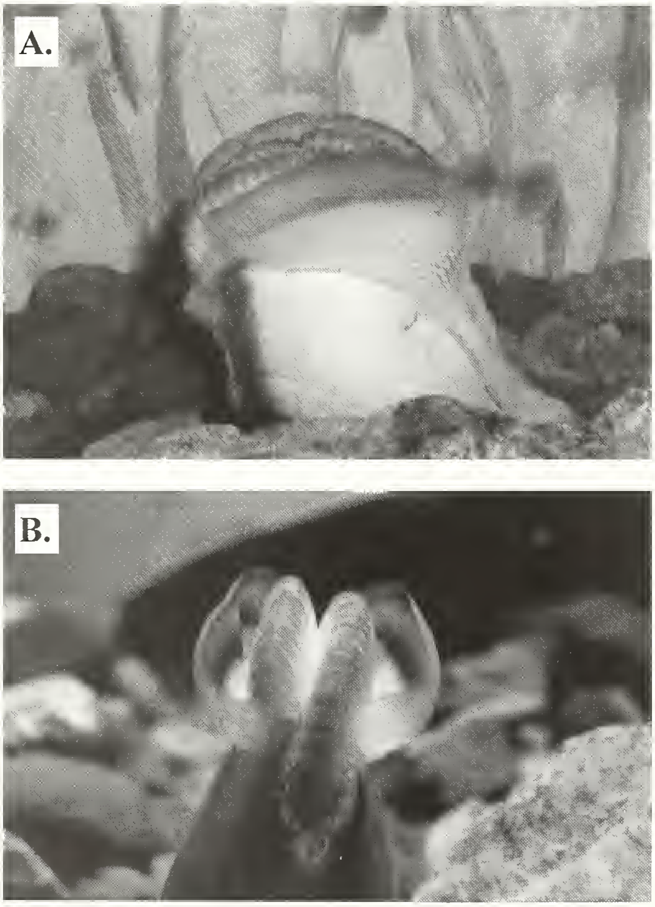

Figure 1. A, Lateral and B, anterior view of the mantle-flap lure of *Lampsilis teres*. Photographs (© 2008 by Paul Frese) reproduced with the permission of the copyright holder.

Fish encounters

Fish were observed within the immediate vicinity of the mussels frequently. The fishes included bluegill (*Lepomis macrochirus*), longear sunfish (*Lepomis megalotis*), and largemouth bass (*Micropterus salmoides*). Relatively large numbers of small longear and bluegill sunfish were seen during both day and night visits, but a largemouth bass ($N = 1$, ~115 cm total length) was encountered only once, at night. The bass approached a mantle-lure in full display and struck it. Following the strike, the bass retreated for 2-5 s and swam off. The mussel continued to display immediately following the strike and rested only after the bass had left. Although small bluegill and longear sunfish were present during all visits, there were no attacks by these fish on the displaying lures although these fish would often pay close attention to a lure in full display.

DISCUSSION

Display timing

The observation that *Lampsilis teres* displays occurred only at night indicates that daytime is not an effective time to attract a suitable fish host. These observations provide further documentation that lure displays vary with time of day and presence of suitable fish hosts (Haag and Warren 2000). Many centrarchids and a number of other freshwater fishes are known to exhibit diel movements and generally move from more midstream or open-water during the day to littoral habitats at night (Helfman 1993, Shoup *et al.* 2004, Rypel and Mitchell 2007). These movements are coupled with the movements of their prey, many of which are also driven by diel cycle (Helfman 1993, Layman and Winemiller 2004). Sunfishes were observed in numbers around the displaying mussels at night and are known to be important prey for adult largemouth bass (Cochran and Adelman 1982, Howick and O'Brien 1983, Gabelhouse 1987). By displaying lures during times which maximize fish host encounters, mussels would improve glochidial transmission. The temporal differences in lure display for *L. teres* at this site were presumably a product of diel changes in host fish locations.

Lure morphology

Mussel species that use large predacious fishes as hosts generally display modified mantle-lures which strongly resemble small prey fishes, insects, and aquatic insect larvae (Kraemer 1970, Haag and Warren 2000). Considering the number of small centrarchid fishes consistently present near the mussels and the lure's color and shape, this mantle-lure apparently mimics these small sunfishes. Each mussel's shell color matched the substrate such that the shells are cryptic in sand and gravel. Meanwhile, the mantle-lure was pink, which combined with the palpitating motions, accentuated the lure's motion underwater, apparently to attract fishes.

Fish encounters

A largemouth bass biting the *Lampsilis teres* mantle-lure demonstrates that the lure is effective at attracting a suitable fish host (Fuller 1974, Watters 1994). In other trips to this site, I have also collected black crappie and warmouth, both of which are reported as fish hosts for this mussel (Watters 1994). Channel catfish (*Ictalurus punctatus*), flathead catfish (*Pylodictis olivaris*), freshwater drum (*Aplodinotus grunniens*), smallmouth buffalo (*Ictiobus bubalus*), spotted bass (*Micropterus punctulatus*), and spotted gar (*Lepisosteus oculatus*) were also collected, although they are currently not believed to be hosts for *L. teres*. Fitness of *L. teres*, and possibly other nighttime mantle-lure displaying mussels, could be tied to diel movements of fishes. Bluegill and longear sunfish, the other fishes consistently observed near the vicinity of the lures, have not yet been identified as fish hosts for *L. teres* (Watters 1994, Parmalee and Bogan 1998). However, the fact that they are not listed as hosts does not preclude their potential as a host under certain environmental conditions. If one species of *Lepomis* were a host, another species within the genus can, at times, also serve as a host (Haag and Warren 2003). These observations corroborate previous reports of freshwater unionids utilizing nighttime displays to attract fish hosts (Haag and Warren 2000, Toomey et al. 2002) and suggest that nighttime observations may provide information on display behavior in mussel species that have not been encountered displaying during daytime.

Additional field observations on the diel nature of other freshwater mussel species are necessary. If night were a critical display period for other unionids, then such observations would be critical to future conservation efforts such as captive breeding programs. Field observations might reveal primary hosts, especially if the host fishes are nocturnal, cryptic, or rare, and could generate new data and questions regarding the ecology of mantle-flap lures. Fish host identification is often based on a "shotgun approach" involving laboratory infestation tests on a variety of sympatric and common fishes suspected to be hosts. However, lists of potential fish hosts may be inadequate, especially if we ignore fish-mussel encounters occurring at night. The ecology of nocturnal freshwater fishes is not understood well and the diel movements of even well-studied fishes have gone somewhat underappreciated until only recently (Shoup *et al.* 2004, Rypel and Mitchell 2007). Future research on the diel ecology of freshwater mussels will be similarly necessary to develop a more robust understanding of unionids.

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Meta-analysis of the relationship between salinity and molluscs in tidal river estuaries of southwest Florida, U.S.A.

Paul A. Montagna[1], Ernest D. Estevez[2], Terry A. Palmer[1], and Michael S. Flannery[3]

[1] Harte Research Institute for Gulf of Mexico Studies, Texas A&M University - Corpus Christi, 6300 Ocean Drive, Unit 5869, Corpus Christi, Texas 78412-5869, U.S.A., Paul.Montagna@tamucc.edu

[2] Mote Marine Laboratory, 1600 Ken Thompson Parkway, Sarasota, Florida 34236, U.S.A.

[3] Southwest Florida Water Management District, 2379 Broad Street, Brooksville, Florida 34604, U.S.A.

Abstract: The estuaries and rivers of the western coast of Florida have been under intense study for some time to identify relationships between inflows, salinity, and natural resources. The molluscs have been shown to be especially sensitive to salinity in other parts of the world. The current study performed a meta-analysis of existing data sets of southwest Florida mollusc communities to identify salinity-mollusc relationships at regional scales. The mollusc species are controlled more by water rather than the sediment they live in or on. The most important variable correlated with mollusc communities was salinity, which is a proxy for freshwater inflow. Although total mollusc abundance was not a good indicator of inflow effects, certain indicator species characterized salinity zones in southwest Florida rivers. *Corbicula fluminea* (Müller, 1774), *Rangia cuneata* (Sowerby, 1831), and *Neritina usnea* (Roding, 1798) were the only common species that occurred in the oligohaline zone at salinities below 1 psu. Although *C. fluminea* was the best indicator of freshwater habitat, it is a non-native, invasive bivalve species. The bivalve *R. cuneata* is an indicator of mesohaline salinity zones with an estimated tolerance of up to 20 psu. The gastropod *N. usnea* is also common in fresh to brackish-water salinities. *Polymesoda caroliniana* (Bosc, 1801) was present at salinities between 1 and 20 psu, which span the oligohaline and mesohaline zones. *Tagelus plebeius* (Lightfoot, 1786), *Crassostrea virginica* (Gmelin, 1791), *Mulinia lateralis* (Say, 1822), *Littoraria irrorata* (Say, 1822), and *Ischadium recurvum* (Rafinesque, 1820) are also good indicators for polyhaline salinity zones. These salinity ranges can be used to predict changes in mollusc assemblages in response to alterations in salinity that result from actual or simulated changes in freshwater inflow.

Key words: Mollusca, benthos, freshwater inflow, indicator species, water management

Estuaries are among the most productive environments on Earth (Odum 1959). The mixing of freshwater with seawater is the defining characteristic of an estuary, and thus, there is much interest in how alterations of freshwater inflow patterns might affect estuarine productivity (Montagna *et al.* 2002b). Certainly, the increasing size of the human footprint has had a dramatic effect on altering the courses and characteristics of rivers, streams, and lakes; these watershed-level changes have had effects on downstream estuaries in the west (Kimmerer 2002) and Gulf of Mexico coasts (Alber 2002, Powell *et al.* 2002) of the U.S.A. To identify the effects of altered flow, ecological indicators must be developed. Molluscs are ideal organisms to indicate inflow effects because of their life habits and feeding modes (Estevez 2002). Molluscs have well-defined relationships between species distributions and physicochemical variables that are affected by freshwater inflows, *e.g.* salinity (Montagna and Kalke 1995). Filter or suspension-feeding molluscs also depend on primary productivity in the water column for food, which is also affected by nutrients carried by freshwater inflow into estuaries.

The Mote Marine Laboratory (MML) and the Southwest Florida Water Management District have completed studies of mollusc distributions for six tidal rivers in southwest Florida located between the Springs Coast, Charlotte Harbor, and Tampa Bay (Fig. 1). A consistent methodology was used in these studies for the Peace River, Alafia River, Myakka River, Weeki Wachee River, Shell Creek, and the Shakett Creek Dona/Roberts Bay system (MML 2002, 2003, Estevez 2004a, 2004b, 2005). Extensive environmental data also exists for freshwater inflows and physicochemical variables (*e.g.*, salinity, dissolved oxygen, pH, and sediment characteristics) in these systems that cover the period of mollusc data collection. Although there have been studies of individual river and creek systems in Florida, there has not been an effort to combine data from many tidal rivers to quantify factors that affect mollusc distributions at the regional scale. Understanding the relationships between salinity and other environmental parameters that relate to mollusc distributions is important to evaluate the freshwater flow requirements needed to protect the natural resources in coastal ecosystems.

The overall goal of the current study was to (1) identify indicator species of freshwater inflow effects and (2) to better define the physical and chemical requirements of mollusc species that inhabit tidal river systems in southwest Florida.

The purpose was to synthesize existing information on the relationships between freshwater inflows and the distribution of mollusc populations among the tidal rivers of southwest Florida. The approach used in this project was to organize the mollusc and environmental data from the six tidal river systems into one database with a common format, to find the appropriate spatial scales in the data so that the different tidal rivers could be compared, and to perform a multivariate analysis on the combined data sets.

MATERIALS AND METHODS

Study area

The study sites were all located on the west coast of peninsular Florida (Fig. 1). They group into four areas of the coast: Weeki Wachee River estuary, Alafia River in Tampa Bay, Curry Creek and Shakett Creek located in the Dona/Roberts Bay estuary, and Charlotte Harbor estuary.

Charlotte Harbor bay and estuary complex contained six of the 10 sites studied, and four of the six were in the arm of Charlotte Harbor that is dominated by the Myakka River. There were three sites that were connected to the Myakka River: (1) Big Slough is near the 14 km marker, (2) Deer Prairie Creek is near the 19 km marker, and (3) Blackburn Canal is near the 32 km marker. The eastern arm of Charlotte Harbor is dominated by the Peace River, which is connected to Shell Creek near the 15 km marker. The Peace River ecosystem has been sampled three times: twice in the Peace River itself and once just in Shell Creek.

Shakett and Curry Creeks are located in the Dona/Roberts Bay complex in the region designated as the Venice Estuary. This area is north of, but adjacent to, the Charlotte Harbor estuary. Shakett Creek ends in Dona Bay and Curry Creek ends in Roberts Bay.

The Alafia River is about 80 km long and drains into Tampa Bay. Further north are the two small tidal rivers: the Weeki Wachee and the Mud Rivers. The Weeki Wachee River is a small, spring-fed system in which the penetration of brackish water is generally less than 2.5 km upstream from the river mouth. Mud River, which is also spring-fed, joins the Weeki Wachee about 1.4 km upstream of the river mouth. While the upstream reaches of the Weeki Wachee are fresh, the Mud River receives flow from brackish springs and salinity in the Mud River increases upstream toward the river head.

Mollusc data

Data for a meta-analysis on molluscs were extracted from several reports designed and implemented by the Mote Marine Laboratory (MML) (MML 2002, 2003, Estevez 2004a, 2004b, 2005). The data sets were complex and had to

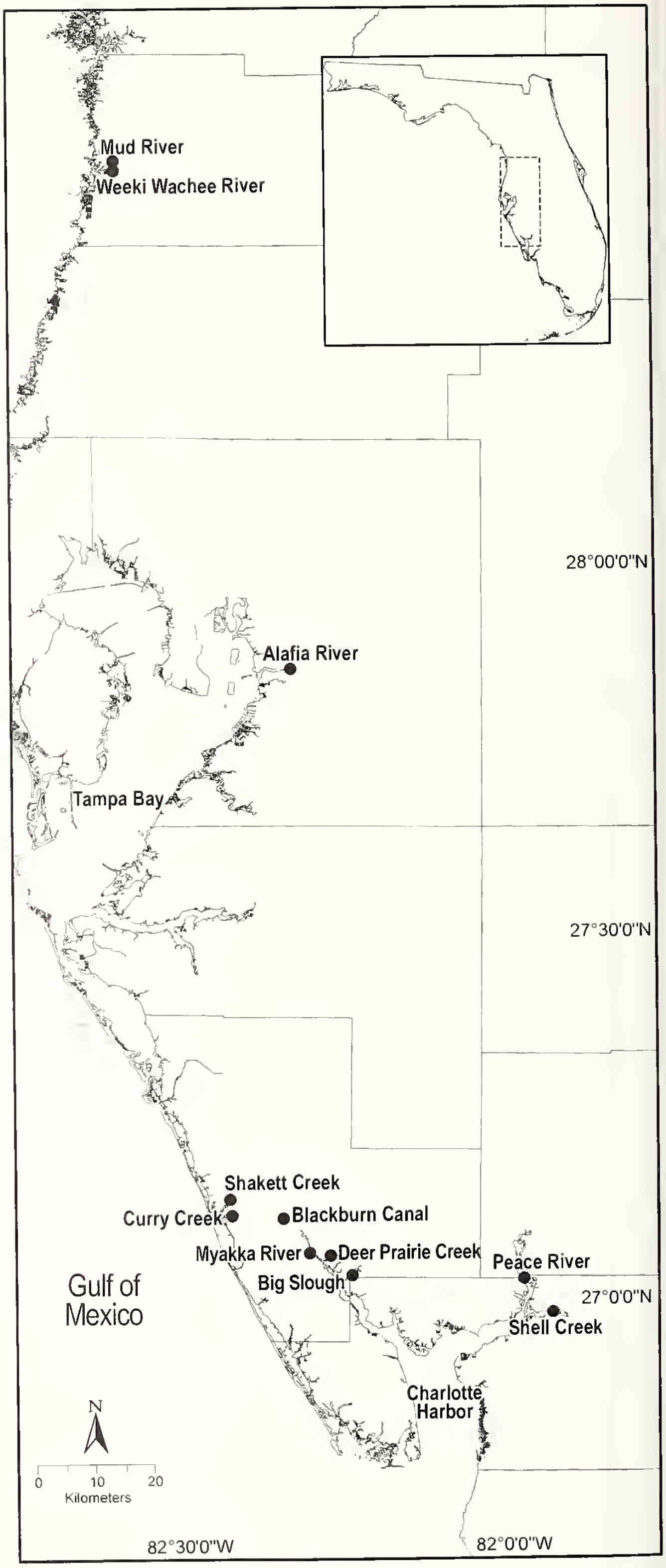

Figure 1. Map of the west coast of Florida showing the study sites.

be concatenated, merged, and formatted prior to analysis. The initial steps in database creation were to determine the relationship between site designations in the data set, if there were any differences in the actual sampling designs in the different rivers, and if there were aggregation relationships among the rivers (Table 1).

The sampling design employed by MML consists of molluscs being sampled along transects within each river system. The transects run lengthwise originating at the mouth of each river, heading upstream; hence, distance and station number names increase with freshwater influence. The original data sets varied uniquely among river systems; however, all samples were characterized by distance along the river transect and the mollusc species composition. These distances represented the stations within the river site, and a total of 180 such stations were sampled across all sites (Appendix 1). At each sampling location, molluscs were sampled systematically across the river channel perpendicular to the river centerline so that samples were collected from all major habitats found in mid-channel, shallow subtidal, and intertidal areas. Most sampling locations were spaced at half-kilometer intervals.

For each sampling event, the variables reported included the number of juvenile molluscs, the number of live molluscs, the number of dead molluscs, the size of shells, and whether the samples were taken from the subtidal or intertidal area of the river system. For all statistical analyses in the current study, mollusc counts from the subtidal and intertidal zones of each station were combined. Sample area was 0.464 m^2, and the raw counts were converted to abundance of individuals per square meter ($n\ m^{-2}$) for all univariate and multivariate analyses. For the current study, meta-analysis was focused on live molluscs; however, the dead shells do provide information on historical communities.

Samples from multiple years of sampling were found only from the Peace River (Table 1). For the purpose of the current study, the sampling stations at Peace River were averaged over the two years they were sampled (1999 and 2000). Combining the two years of data was supported in part by the absence of evidence for shell drift.

To enable a meta-analysis that simultaneously compares all rivers using multivariate methods, the distance along each transect had to be standardized. To do this, the distance from each river's mouth of each sampling station was aggregated into 2-km segment bins (Appendix 1). This was performed by rounding the actual distance from the mouth of the river (in kilometers) to increments of two. Each segment was numbered as the midpoint of the actual distance, thus a segment labeled 2 km would encompass stations found at 1.0 km to 2.9 km of a transect. Overall, 67 new stations, or 2-km segments, were created for analysis. Because more than one sampling station occurred within many new 2-km segments, species abundances were averaged across stations within each new 2-km segment prior to analysis to ensure a balanced sampling design.

The scientific names of all the species were verified and made consistent across all data sets. In addition, the full taxonomic description was verified. The convention for species names and taxonomy used in the current study is based on the Species 2000 and Integrated Taxonomic Information System (ITIS) Catalogue of Life: 2006 Annual Checklist (Bisby *et al.* 2006, http://www.sp2000.org).

Hill's number one (N1) diversity index was used to report species diversity (Hill 1973). Hill's N1 is the exponential form ($e^{H'}$) of the Shannon-Weaver diversity index H'. N1 was used because it has units of numbers of dominant species, and it is easier to interpret than most other diversity indices (Ludwig and Reynolds 1988).

Multivariate analyses

Community structure of mollusc species was analyzed by non-metric multi-dimensional scaling (MDS). All multi-

Table 1. Location of sites within river systems, sampling year(s), and time period that water hydrography data were collected.

Estuary	River system	Site (or creek)	Molluscs	Hydrography
Tampa Bay	Alafia	Alafia	2001	Jan 1999-Dec 2003
Charlotte Harbor	Myakka	Big Slough	2004	—
Charlotte Harbor	Myakka	Blackburn	2004	—
Charlotte Harbor	Myakka	Deer Prairie	2004	—
Charlotte Harbor	Myakka	Myakka	2004	Feb 1998-Mar 2005
Charlotte Harbor	Peace	Peace	1999 & 2000	Aug 1996-Dec 2004
Charlotte Harbor	Peace	Shell	2004	Feb 1991-Dec 2004
Venice	Dona/Roberts Bay	Curry	2004	Aug 2003-May 2005
Venice	Dona/Roberts Bay	Shakett	2004	Aug 2003-May 2005
Weeki Wachee	Weeki Wachee	Mud River	2005	July 2003-May 2005
Weeki Wachee	Weeki Wachee	Weeki Wachee	2005	July 2003-May 2005

variate statistical analyses were performed using Primer software (Clarke and Warwick 2001). The MDS procedure was used to compare mean abundances of individuals of each species for each river-site-segment combination. The MDS analysis was completed using a Bray-Curtis similarity matrix on log-transformed ln (x + 1) data. The distance between river-site-segment combinations in the MDS plot can be related to community similarities or differences between rivers, sites, and segments. Differences and similarities among communities were highlighted based on cluster analysis calculated from the similarity matrix. A subset of species that represented the spatial pattern in an MDS plot was determined using the BVSTEP procedure. The BVSTEP procedure employs a step-wise approach to determine the minimum subset of species that can yield the same pattern of community structure obtained from the entire data set (Clarke and Warwick 1998).

Physicochemical variables

Physicochemical data for each tidal river system included profiles of temperature, dissolved oxygen, salinity, and pH taken along all transects. Profiles were measured at various distances along the transects in each river on multiple dates over a period of 2-13 years. The length of period and actual years sampled varied for each river (Table 1). As with the mollusc data, the distance along each transect was converted into the same 2-km segments for the physical data. The four water hydrography parameters measured (temperature, dissolved oxygen, salinity, and pH) were all averaged by transect segment and river.

Principle Components Analysis (PCA), a parametric multivariate method, was used to determine differences for the environmental measurements among river-segment combinations. As with MDS, the distance between river-segment combinations in the PCA plot can be related to actual similarities or differences in water hydrography between river-segment combinations.

Sediment

Samples along each transect were also analyzed by MML for sediment characteristics. Sediment grain size distributions (median, mean, % sand, % silt, % clay, skewness, kurtosis), sediment moisture, and the proportion of organic material present in the sediment were measured.

Relating molluscs and environmental factors

Relationships between mollusc communities and environmental factors were investigated using the Biota-Environment (BIO-ENV) procedure using Primer software (Clarke and Warwick 2001). The BIO-ENV procedure is a multivariate method that matches biotic (*i.e.*, mollusc community structure) with environmental variables. This is carried out by calculating weighted Spearman rank correlations (ρ_w) between sample ordinations of all environmental variables and biotic variables (Clarke and Ainsworth 1993). Correlations are then compared to determine the best match. The BIO-ENV procedure uses different numbers of abiotic variables in calculating correlations to investigate the different levels of environmental complexity. For this study, the mollusc species abundance MDS ordination was compared with all physicochemical and sediment variables. A total of 49 of the 67 river-segment combinations were used in the multivariate analysis because these stations had all sediment, physiochemical, and mollusc data necessary for analysis. The significance of relationships were tested using RELATE, a non-parametric form of the Mantel test. The BIO-ENV and RELATE procedures were calculated with Primer software (Clarke and Warwick 2001).

Salinity was used as a proxy for distance from a freshwater source because salinity increases as distance from the freshwater source increases. Salinity was directly compared with individual species abundances, total mollusc abundances, and mollusc diversity.

The relationship between mollusc abundance, diversity, and salinity were examined with a non-linear model, which was used successfully in Texas estuaries (Montagna *et al.* 2002a). The assumption behind the model is that there is an optimal range for salinity and values decline prior to and after meeting this maximum value. That is, the relationship resembles a bell-shaped curve. The shape of this curve can be predicted with a three-parameter, log normal model:

$$Y = a \times \exp(-0.5 \times (\ln(X / c) / b)^2)$$

The model was used to characterize the nonlinear relationship between a biological characteristic (Y, *e.g.*, abundance or diversity) and salinity (X). The three parameters characterize different attributes of the curve, where a is the peak abundance value, b is the skewness or rate of change of the response as a function of salinity, and c the location of the peak response value on the salinity axis (Montagna *et al.* 2002a). The model was fit to data using the Regression Wizard in SigmaPlot (version 10) which uses the Marquardt-Levenberg algorithm to find coefficients (parameters) of the independent variables that give the best fit between the equation and the data (Systat 2006).

RESULTS

Physical environments

With the exception of Mud River, salinity decreased with distance from the river or creek mouth in all the river systems (Fig. 2). Because rivers and transects in each river were different, the length of each transect covered different

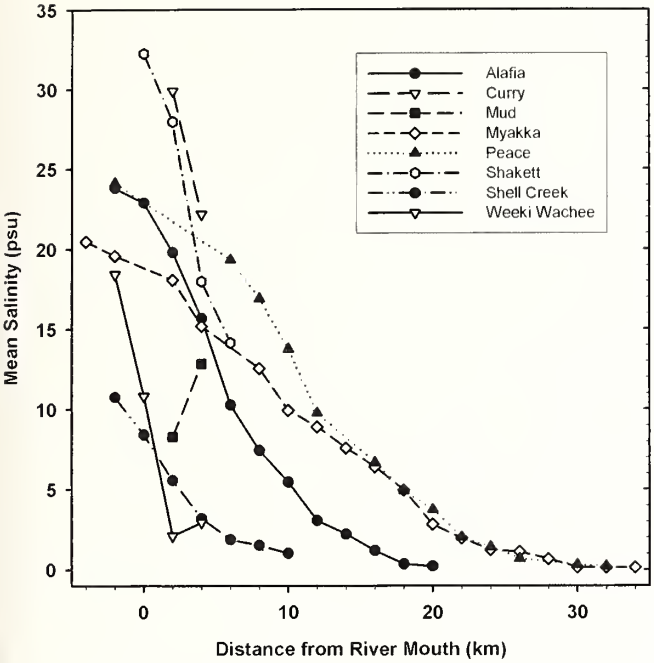

Figure 2. Mean salinity along transects at each creek /site system.

salinity ranges; thus, a km segment number in one river did not correspond to a similar salinity range in another system. The transects of the Alafia, Myakka, and Peace Rivers were at least 20 km long and had mean salinity ranges between 20 and 25 psu. Although the Shakett Creek and Weeki Wachee River transects covered less than 8 km, they also covered a mean salinity range of at least 15 psu. The transects in Curry Creek, Shakett Creek, and Mud River did not extend to freshwater, as did the transects on the other river systems. A salinity barrier on Shakett Creek truncates this river and structurally isolates a freshwater zone under most flow conditions. As described earlier, the Mud River is an unusual system that is fed by brackish springs and salinity increases toward the river head. Only two transect segments were sampled in each of Curry Creek and the Mud River.

The principal components (PC) analysis reduced the four environmental variables of salinity, temperature, pH, and dissolved oxygen (DO) into two PC axes. The first (PC1) and second (PC2) principal components of the physicochemical data explained 98.7% and 0.7% of the variation within the data set, respectively (total 99.4%; Fig. 3). PC1 was dominated by salinity and pH differences and PC2 by temperature (Fig. 3A). Dissolved oxygen differences were not important because it varied little from the origin. Thus, PC1 represents changes over distance along the transects or between rivers, and PC2 represents water body and temporal change, with higher temperatures as higher PC2 values. The PC analysis demonstrates that Alafia, Weeki Wachee, Sha-

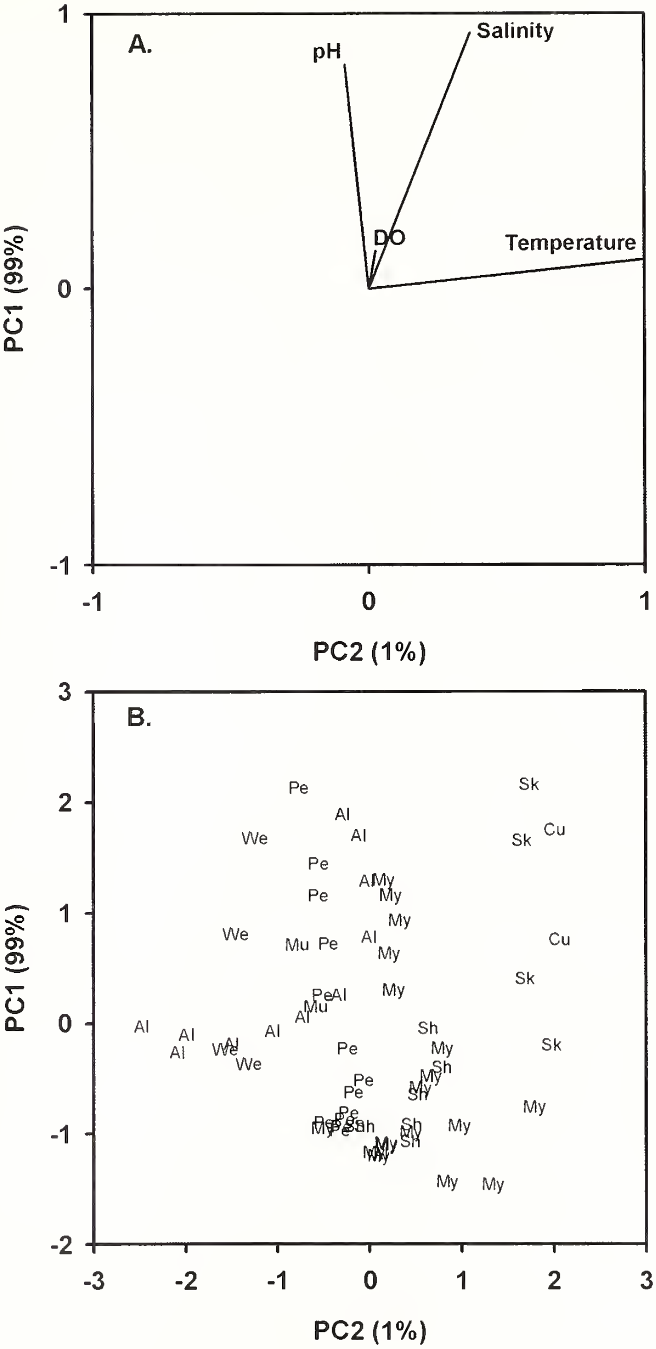

Figure 3. Principal Components Analysis (PCA) of water hydrography in southwest Florida rivers. A, Principal Component variable loadings. B, Transect segment-river station scores. Symbol key: Al, Alafia River; Cu, Curry Creek; Do, Dona/Roberts Bay; My, Myakka River; Pe, Peace River; Sk, Shakett Creek; Sh, Shell Creek; We, Weeki Wachee River.

kett, Curry, and Myakka are all distinct water bodies (Fig. 3B). The differences are primarily a result of separation along the PC2 axis. Shakett, Curry, and Myakka had similar temperature conditions but were distinct from the Alafia and Weeki Wachee in this regard. Separation along PC1 and PC2 indicates the Peace and Myakka Rivers were very similar to one another with respect to their physical characteristics. The Alafia River had a unique pattern where at low salinities temperatures increased, but at high salinities temperatures were similar.

Taphonomy

Examining the fossil shells or death-assemblages, *i.e.*, taphonomy, is a good technique to understand the derivation of extant benthic communities because it is an indicator of the living community prior to sampling and between sampling occasions (Powell *et al.* 1986). The total abundance was similar with a mean of 95 m^{-2} relict shells compared to a mean of 82 m^{-2} live shells. The proportion of dead shells to live shells was similar overall because a paired-difference test was not significantly different ($P = 0.7822$). A total of 56 relict species were found (Appendix 2). However, 22 more species were found among relict shells than live shells. This does not mean that species have gone extinct or are no longer found in the study area. Shells can be transported after death, and the age of the shells are unknown; therefore, the remainder of this current report focuses on the living fauna. However, there was no evidence from field observations that shells were transported in these low-flow rivers and creeks.

Mollusc community structure

A total of 33 live species were found in all of the rivers sampled (Appendix 2). Of these, 25 species were bivalves and eight species were gastropods. Two families of bivalves, Tellinidae and Mytilidae, were represented by four species each, and there were three species of Veneridae. Otherwise, all families were represented by only one or two species.

The dominant species was the Asian Clam *Corbicula fluminea* (Müller, 1774) an exotic species introduced to Florida waters (Appendix 3). The large number of *C. fluminea* was due to very high densities of this species in the tidal freshwater reaches of the Peace River; a lower density was found in the Myakka River, and five rivers had none. *Corbicula fluminea* was found in 27 river-segments and had a mean density of 33 individuals m^{-2} throughout all 67 river-segments. This represented 40% of total mean abundance. The next four most dominant species were *Polymesoda caroliniana* (Bosc, 1801; 11%), *Rangia cuneata* (Sowerby, 1831; 8%), *Tagelus plebeius* (Lightfoot, 1786; 6%), and *Amygdalum papyrium* (Conrad, 1846; 5%). These top five most abundant molluscs were bivalves and comprised 70% of all specimens found. The dominant gastropod *Neritina usnea* (Roding, 1798) was the sixth-ranked species in dominance (4% of total mean abundance). The second-most dominant species *P. caroliniana* was found in 35 river-segments.

Dominance patterns were different in different rivers (Appendix 3). For example, *Corbicula fluminea* was dominant only in the Peace and Myakka Rivers. In contrast, *P. caroliniana* was dominant in Shell Creek and Big Slough, and the second-most dominant species in Deer Prairie Creek, Myakka, and Weeki Wachee Rivers. *Rangia cuneata* was dominant in Deer Prairie and was the only organism found in Blackburn Canal. *Tagelus plebeius* was co-dominant in Weeki Wachee and the dominant species in Mud River and Curry Creek. *Geukensia granosissima* (Sowerby, 1914) was dominant in the Alafia River, and *Crassostrea virginica* (Gmelin, 1791) was co-dominant in Weeki Wachee and dominant in Shakett Creek. However, the distribution of *C. virginica* in the Weeki Wachee River was largely limited to individuals located near the river mouth.

Similarity in mollusc communities among the river-segment sites is generally low (Fig. 4). All of the river-segment combinations are found in associations of groups of

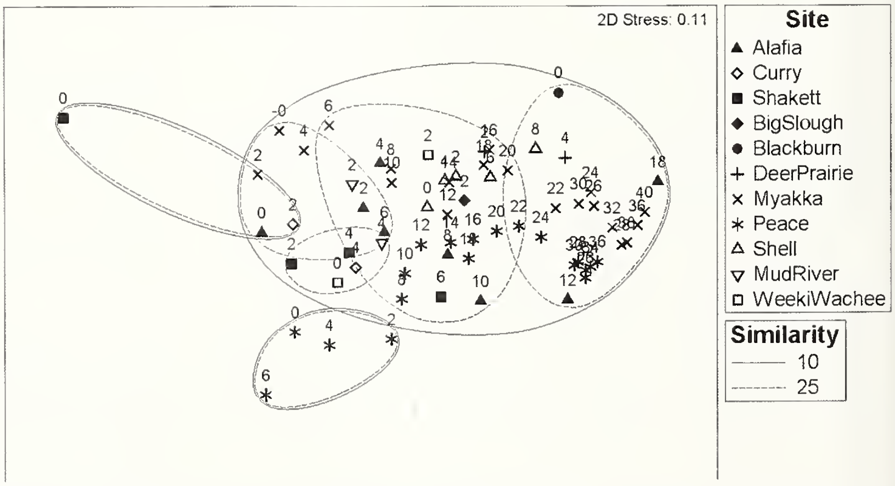

Figure 4. Relationships between mollusc communities from multi-dimensional scaling (MDS) analysis. Symbols represent the river or creek site with shape and color, and the km segment number is listed above the river symbol. Segment 16 from the Alafia River is outside the range of this plot. Similarity is indicated with lines drawn around points.

no more than 10% similarity. At the 10% similarity level there are three groups, two smaller groups with low station numbers (*i.e.*, more marine conditions) and one large group. At the 25% similarity level, the large group splits into 4 smaller groups. Although the pattern of river-segment groupings is based on 33 species, it is being driven by just seven species: *Corbicula fluminea*, *Crassostrea virginica*, *Littoraria irrorata* (Say, 1822), *Neritina usnea*, *Polymesoda caroliniana*, *Rangia cuneata*, and *Tagelus plebeius* (BVSTEP, rho > 0.95, r = 0.96). These species drive the trend in which downstream segments close to marine sources (with low 2-km segment numbers) tend to group to the left, while higher segment numbers groups to the right in the MDS plot (Fig. 4).

The four groups at the 25% level within the large central group at the 10% similarity level (Fig. 4) can be explained based on the distribution of three species. From left to right in Fig. 4, the station groups are dominated by *Crassostrea virginica*, *Polymesoda caroliniana*, and *Corbicula fluminea*. Two groups fell outside the 10% similarity level. One group had four Peace River segments (0, 2, 4, and 6). The other group had just one Shakett Creek 0 segment, and this was most different from all other segments because it had only two rare species: *Chione cancellata* (Linnaeus, 1767) and *Cyclinella tenuis* (Recluz, 1852). The 16-km segment of the transect in the Alafia River was so different from all others that it is not included in the MDS plot. This station is 100% different from all of the other stations sampled, because the station had only one mollusc present, an unidentified snail of the family Planorbidae, which was not found in any of the other river systems.

Mollusc-environment relationships

Two approaches are used to relate molluscs to the environment, but in all cases salinity is used as the surrogate for inflow. One approach is to relate (by univariate or multivariate models) salinity with abundance, diversity, or community structure. The second approach is to examine the relationship between abundance and salinity to identify those species or species groups that might have optimal salinity ranges.

For the first approach, a non-parametric multivariate analysis procedure (BIO-ENV) was used to identify the combinations of environmental variables that could best predict mollusc abundance. Out of 62 transect-segments sampled for water hydrography and 67 transect-segments sampled for molluscs, there were only 45 common transect-segments that could be analyzed using BIO-ENV because of missing water hydrography data in the other transect-segments. Salinity, temperature, and pH were the environmental variables that correlated the highest with the mollusc community distributions ($\rho_w = 0.612$). The RELATE procedure indicated that this correlation was significant ($P < 0.001$). The single physical variable that correlated the highest with mollusc communities was salinity ($\rho_w = 0.566$). In fact, salinity was the only variable that fit the community distributions in all the tests. The water hydrography variables had higher correlations with the mollusc communities than any single, or combination of, sediment characteristics. Of the sediment variables, median and mean grain size fit best, but all sediment variables were selected after salinity, temperature, and pH. This indicates that overlying water properties, especially salinity values, have more control on the mollusc communities than the sediment characteristics.

In the second approach, total mollusc abundance did not correlate with salinity among all rivers. The highest abundances occurred at low salinities, but this is attributed to the large population of *Corbicula fluminea* that occurred in the Peace River at low salinities. Mollusc diversity increased with salinity, particularly as salinity increased from 0 to 2 psu, but the correlation was weak. Hill's N1 values were consistently close to one where mean salinity was close to one; however, as salinity and overall N1 increased, so too did the range of N1 values.

Two rivers, the Myakka and Peace, were sampled across long transects (Fig. 2). Examining distributions along salinity gradients in these two rivers separately avoids bias due to differences between the systems. In both rivers there were strong relationships between both diversity and abundance with salinity where abundance and diversity increased with increasing salinity, then peaked, before declining (Fig. 5). This response emulates a 3-parameter log-normal distribution, which was found to fit total macrofauna abundance in a Texas estuary (Montagna *et al.* 2002a). The nonlinear relationship between salinity and diversity was stronger in the Peace River than the Myakka River, based on the probability levels (P) and goodness of fit parameters (R^2) (Table 2).

The ten dominant species were examined for correlations with salinity (Table 3). *Corbicula fluminea* was found only where mean salinities were <7 psu, but it was most common where mean salinities were ≤2 psu (Fig. 6A). However, the maximum salinity value (parameter *c* in Table 2) was 0.6 psu. *Corbicula fluminea* occurred at abundances higher than 10 m^{-2} only in the Myakka and Peace Rivers. *Polymesoda caroliniana* was found in all river systems and occurred where salinities ranges from 1 to 20 psu (Fig. 6B) while peaking at 5 psu (Table 2). Both *P. caroliniana* and *C. fluminea* are in the same family (Corbiculidae). *Rangia cuneata* and *Tagelus plebeius* were found in low to moderate salinities and occurred at salinity peaks of 4 and 7 psu respectively (Figs. 6C-D). *Crassostrea virginica* and *Geukensia granosissima* were generally found at higher salinities, as indicated by salinity peaks of 24 and 10 psu, respectively. *Mulinia lateralis* ranged from 5 to 15 psu, and the model cal-

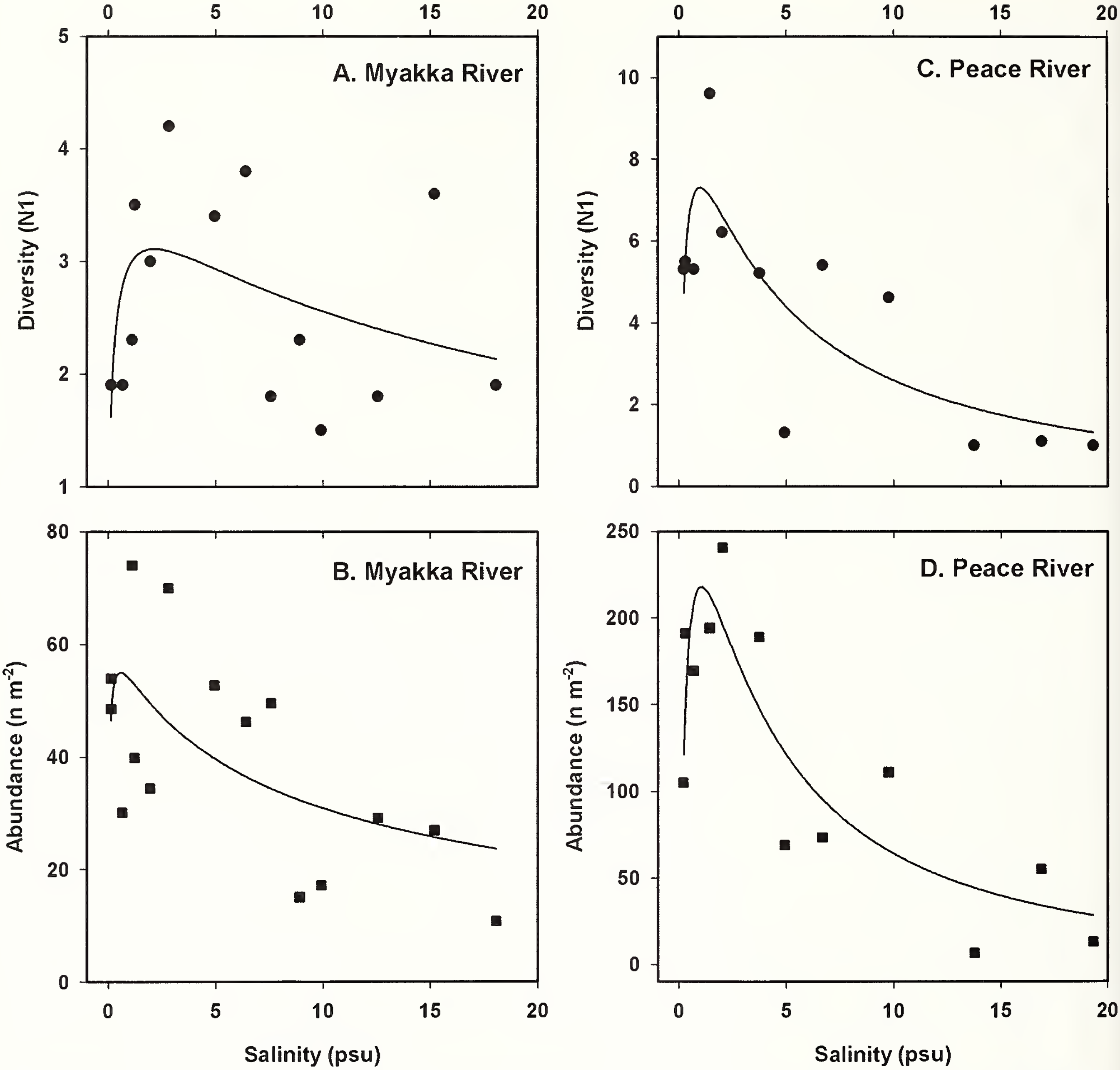

Figure 5. Relationship between total mollusc diversity (A) and abundance (B) vs. salinity at Myakka River and diversity (C) and abundance (D) versus salinity at Peace River. Circles, Hill's N1 diversity index; squares, abundance.

culated a peak at 14 psu. According to the model, *Neritina usnea* abundance did not change with salinity ($P = 0.43$). *Littoraria irrorata* and *Ischadium recurvum* were found over a wide range of salinities, with peak salinities at 14 and 12 psu, respectively. Two other species, *Amygdalum papyrium* and *Tellina versicolor*, occurred in less than 9 segments, precluding an estimation of the salinity range.

DISCUSSION

The overall purpose of this project was to better define the biogeography, community structure, and the physical and chemical requirements of mollusc species that inhabit tidal river systems in southwest Florida. To meet this purpose, an inter-river meta-analysis was performed to examine

Table 2. Parameters from nonlinear regressions to predict mollusc characteristics from salinity. These parameters are represented on lines in Figs. 5 and 6. Probability (P) that model fits the data, percent of variance explained by data (R^2), parameters for maximum biological value (a), rate of change (b), and salinity in which maximum abundance occurs (c), and standard deviation for parameters in parentheses. N1, Hill's diversity index; n, abundance (individuals per m^2); all species are n m^{-2}.

Variable	P	R^2	a	b	c
Myakka N1	0.1658	0.26	3.11 (0.36)	2.45 (0.65)	2.15 (0.86)
Myakka n	0.0682	0.36	54.9 (7.9)	2.63 (0.84)	0.59 (0.41)
Peace N1	0.0098	0.64	7.29 (1.02)	1.61 (0.31)	0.99 (0.28)
Peace n	0.0013	0.77	218 (24.8)	1.44 (0.20)	1.05 (0.20)
Neritina usnea	0.4320	0.03	4.92 (1.71)	2.96 (2.77)	0.45 (1.33)
Corbicula fluminea	0.0001	0.31	178 (43.2)	0.78 (0.19)	0.63 (0.18)
Rangia Cuneata	0.0001	0.38	27.3 (4.8)	0.49 (0.08)	3.69 (0.31)
Polymesoda caroliniana	0.0001	0.32	28.8 (5.1)	0.66 (0.13)	4.89 (0.63)
Tagelus plebeius	0.0003	0.28	15.4 (3.0)	0.48 (0.12)	7.30 (0.90)
Geukensia granosissima	0.0001	0.77	156 (11.9)	0.006 (3e-7)	10.3 (3e-6)
Ischadium recurvum	0.0169	0.16	5.68 (1.81)	0.31 (0.11)	12.3 (1.3)
Mulinia lateralis	0.0001	0.37	324 (53.3)	0.006 (3e-7)	13.6 (8e-6)
Littoraria irrorata	0.0001	0.33	6.43 (1.28)	0.31 (0.07)	13.8 (0.98)
Crassostrea virginica	0.0001	0.33	19.3 (4.2)	0.18 (0.04)	22.4 (1.0)

Table 3. Salinity range of twelve most abundant species.

Species	Salinity range (psu)	Transect segments with species present
Corbicula fluminea	<7 (most ≤2)	20
Polymesoda caroliniana	1 to 20	32
Rangia cuneata	<16 (most ≤10)	23
Tagelus plebeius	>2	25
Geukensia granosissima	10 to 24	5
Amygdalum papyrium	2 to 20	8 (7 in Peace R.)
Crassostrea virginica	>7	13
Mulinia lateralis	>2	10
Neritinia usnea	<18	20
Tellina versicolor	2 to 18	7 (all in Peace R.)
Littoraria irrorata	>2	17
Ischadium recurvum	>6	11

relationships between the distribution of mollusc populations both within and among tidal river estuaries and tidal river locations. The meta-analysis combines independent studies to reach general conclusions (Gurevitch and Hedges 2001). The sampling gear and spatial sampling strategies were consistent for both water hydrography and mollusc data, making this meta-analysis a simple task. Although these data were collected without specific regard to a regional scale design and analysis, the data fit well into a sampling design, even though all samples were not taken in the same year (Table 1). Two exceptions to this lack of synoptic sampling were the Myakka and Dona/Roberts Bay systems. However, all the rivers exhibited distinct changes in their water hydrography characteristics and mollusc community composition along the estuarine gradient. Therefore, analysis of these data provides meaningful information on how environmental factors affect the distribution and abundance of mollusc populations within these tidal river ecosystems.

River systems were strikingly different. The mollusc communities among all the river stations shared <25% species in common. Although sampling occurred over different years, there were community similarities at similar transect segments among rivers. There were upstream clusters, downstream clusters, and larger clusters of intermediate range transects. The segments with the most similar mollusc communities occurred in the most upstream segments of the Peace, Myakka, and Alafia Rivers. These segments had the most stable, and lowest mean salinities with minimal tidal influence. Further downstream, freshwater influence decreased and salinity was more variable, which allowed different species and communities to persist, compared to stable upstream waters. Other factors such as tides, waves, currents, and inshore geomorphology create diverse habitats both within and between estuarine river systems. This increase in physical diversity from upstream to downstream can cause the considerable differences found in mollusc communities along the salinity gradient and among the rivers. The heterogeneity of the salinity regimes is why the river systems share <25% of species in common.

The highest correlations between physical variables and mollusc communities were with salinity. Salinity differences were, thus, more important than sediment differences in regulating mollusc communities in tidal rivers of southwest Florida. The physical variables with the highest correlations

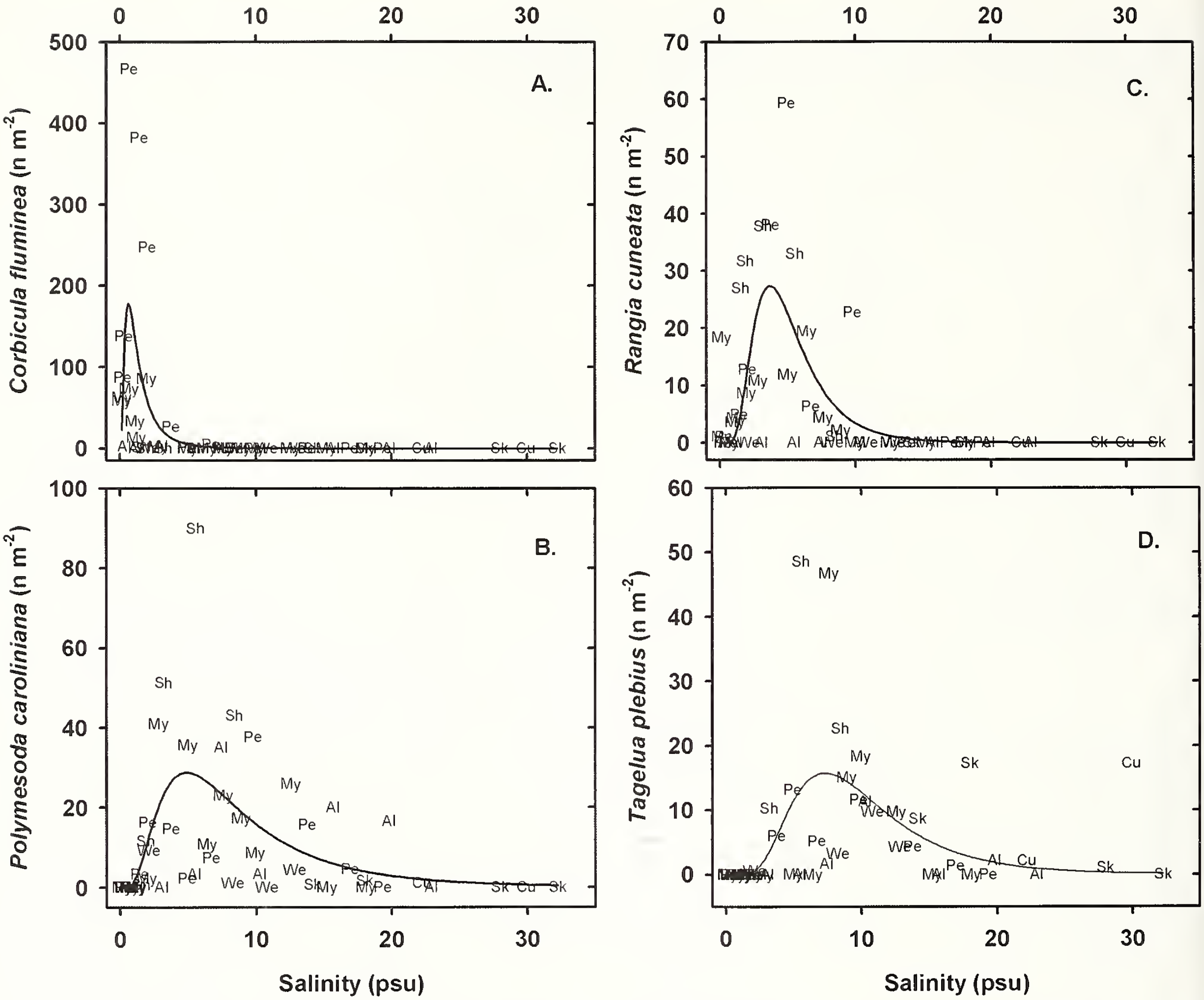

Figure 6. Relationship between salinity and species abundance. A, *Corbicula fluminea*; B, *Polymesoda caroliniana*; C, *Rangia cuneata*; and D, *Tagelus plebeius*. Symbol key: Al, Alafia River; Cu, Curry Creek; Do, Dona/Roberts Bay; My, Myakka River; Pe, Peace River; Sk, Shakett Creek; Sh, Shell Creek; We, Weeki Wachee River.

with the macrofaunal community structure almost always included salinity, temperature and pH. The best single physical indicator of mollusc communities was salinity. Thus, freshwater inflow, which is one factor controlling salinity, is an important factor influencing mollusc community structure and abundance patterns. *Corbicula fluminea*, *Rangia cuneata*, and *Neritina usnea* were the only species common to rivers, creeks, and canals that occurred at salinities below 1 psu. However, *C. fluminea* was the best indicator of freshwater habitat and is an introduced bivalve that can survive salinities up to 13 psu (Morton and Tong 1985) but usually occurs primarily in freshwater (Batelle 2000). *Rangia cuneata* is an indicator of a fresh to brackish-water (Swingle and Bland 1974, Montagna and Kalke 1995). *Neritina usnea* is also common in fresh to brackish-water salinities (Andrews 1992). *Polymesoda caroliniana* is a native, brackish-water bivalve (Gainey and Greenberg 1977) also from the family Corbiculidae. In the current study, *P. caroliniana* was present at salinities between 1 and 20 psu and was present in all creeks/sites.

Tagelus plebeius, *Crassostrea virginica*, *Mulinia lateralis*, *Littoraria irrorata*, and *Ischadium recurvum* are also good indicators for brackish to seawater salinities. The relationship between *C. virginica* and salinity is well known (Turner

2006). *Mulinia lateralis* prefers organically-rich muddy sediments (Grassle *et al.* 1992) and has the ability to survive short periods of anoxia (Shumway *et al.* 1993). *Mulinia lateralis* is typically found in euryhaline habitats (Williams 1984). Although these bivalves are most often found in these brackish to euryhaline salinity zones, they may also be most susceptible to predation in the same area. For example, the oyster drill *Stramonita haemastoma* (Gray, 1839) can severely crop *I. recurvum* and *Rangia cuneata* at high salinities and limit these prey to lower salinity areas along the Gulf of Mexico coast (Brown and Richardson 1988).

Total mollusc abundance and aggregated mollusc species diversity did not indicate freshwater inflow across all rivers, but were useful within rivers. In addition, the trend of transect numbers increasing from left to right in the MDS analysis is evidence of seriation (*i.e.*, linearity or spatial associations) in the mollusc communities.

In summary, from this meta-analysis of southwest Florida communities, mollusc species appear controlled more by water column hydrography rather than the sediment composition. Salinity is the most important environmental variable and is an indicator or proxy for freshwater inflow. One typical approach to link community responses inflow changes is to perform long-term studies of inflow events. The current study used spatial variability at a regional scale to capture a large range of salinity differences, and hence inflow influences. Certain indicator species have been identified that characterize salinity ranges in southwest Florida rivers. These salinity ranges may be useful in predicting mollusc community reactions to changes in freshwater inflow.

Although meta-analysis is an emerging and accepted practice, synoptic sampling over time would greatly improve the ability to accurately determine the relationships between inflow and the mollusc communities, relative to those in other regions. Synchronization of sampling and sample replication would also improve the ability to accurately correlate mollusc communities' response to freshwater inflows using the types of data analysis reported here. Nevertheless, the use of transect-segments in the current meta-analysis and comparing data from the different surveys has led to robust conclusions.

The present study clearly demonstrates that estuarine mollusc species are arrayed along horizontal salinity gradients within tidal river estuaries, with certain species being most common in low salinity zones (*e.g.*, <10-15 psu). In addition to salinity, other factors such as current velocities or the availability of detrital or planktonic food resources could contribute to mollusc distribution patterns in tidal rivers. Low salinity zones are among the habitats that are most vulnerable to impacts and loss within Gulf Coast estuaries because of proximity to human activities in adjacent uplands and the sources of pollution from the contributing watersheds (Lewis and Robison 1995, Beck *et al.* 2005). Low salinity zones are also particularly sensitive to shifts and changes in salinity regimes that could be caused by freshwater withdrawals or salinity intrusions. Given that distinct mollusc communities occur within low salinity waters, the proper management of freshwater inflows and other related watershed activities are very important for maintaining the biological integrity of mollusc populations in tidal river estuaries.

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Appendix 1. Aggregation of Mote Marine Laboratory (MML) sampling data for the current analyses. For each river-site, the new 2-km bin name created and the number of MML stations that were located within the 2-km bin.

River	Site	2-km bin name	Number of stations
Alafia	Alafia	0	2
Alafia	Alafia	2	3
Alafia	Alafia	4	4
Alafia	Alafia	6	4
Alafia	Alafia	8	4
Alafia	Alafia	10	4
Alafia	Alafia	12	3
Alafia	Alafia	16	1
Alafia	Alafia	18	1
Dona/Roberts	Curry	2	3
Dona/Roberts	Curry	4	2
Dona/Roberts	Shakett	0	1
Dona/Roberts	Shakett	2	4
Dona/Roberts	Shakett	4	4
Dona/Roberts	Shakett	6	3
Myakka	Big Slough	2	2
Myakka	Blackburn	0	1
Myakka	Deer Prairie	2	2
Myakka	Deer Prairie	4	1
Myakka	Myakka	−0	2
Myakka	Myakka	2	2
Myakka	Myakka	4	2
Myakka	Myakka	6	2
Myakka	Myakka	8	2
Myakka	Myakka	10	2

Appendix 1. (continued)

River	Site	2-km bin name	Number of stations
Myakka	Myakka	12	2
Myakka	Myakka	14	3
Myakka	Myakka	16	1
Myakka	Myakka	18	2
Myakka	Myakka	20	3
Myakka	Myakka	22	2
Myakka	Myakka	24	1
Myakka	Myakka	26	3
Myakka	Myakka	28	2
Myakka	Myakka	30	2
Myakka	Myakka	32	2
Myakka	Myakka	36	2
Myakka	Myakka	38	3
Myakka	Myakka	40	1
Peace	Peace	0	1
Peace	Peace	2	1
Peace	Peace	4	1
Peace	Peace	6	1
Peace	Peace	8	4
Peace	Peace	10	4
Peace	Peace	12	4
Peace	Peace	14	4
Peace	Peace	16	5
Peace	Peace	18	5
Peace	Peace	20	4
Peace	Peace	22	5
Peace	Peace	24	4
Peace	Peace	26	5
Peace	Peace	28	4
Peace	Peace	30	4
Peace	Peace	32	4
Peace	Peace	34	3
Peace	Peace	36	1
Shell	Shell	0	2
Shell	Shell	2	4
Shell	Shell	4	4
Shell	Shell	6	3
Shell	Shell	8	4
Weeki Wachee	Mud River	2	2
Weeki Wachee	Mud River	4	1
Weeki Wachee	Weeki Wachee	0	2
Weeki Wachee	Weeki Wachee	2	4
Total number of segment bins and stations		67	180

Appendix 2. Taxonomic list of all live and relict species found. Abundance of all relict and live individuals found per m^2 averaged over all samples (*i.e.*, river-site-segment combinations). Abbreviations: CL, Class; OR, Order; and FA, Family.

CL OR FA Species	Dead	Live
Gastropoda		
Pulmonata		
Ellobium		
Melampus sp.	0.055	0
Basommatophora		
Planorbidae		
Planorbidae (unidentified)	0.208	0.032
Neotaenioglossa		
Littorinidae		
Littoraria irrorata (Say, 1822)	0.469	1.811
Epitoniidae		
Epitonium rupicola (Kurtz, 1860)	0.031	0
Calyptraeidae		
Crepidula fornicata (Linnaeus, 1758)	0.318	0
Naticidae		
Polinices duplicatus (Say, 1822)	0.133	0.048
Cerithiidae		
Cerithium atratum (Born, 1778)	0.495	0
Triphoridae		
Triphora melanura (Adams, 1850)	0.031	0
Cephalaspidea		
Bullidae		
Bulla striata (Bruguiere, 1792)	0.073	0
Haminoeidae		
Haminoea succinea (Conrad, 1846)	0.851	1.062
Neogastropoda		
Conidae		
Conus sp.	0.010	0
Nassariidae		
Nassarius vibex (Say, 1822)	2.944	1.395
Melongenidae		
Melongena corona (Gmelin, 1791)	0.247	0.153
Muricidae		
Eupleura sp.	0.021	0
Urosalpinx tampaensis (Conrad, 1846)	0.042	0
Neritopsina		
Neritidae		
Neritina usnea (Roding, 1798)	5.990	3.028
Bivalvia		
Myoida		
Pholadidae		
Cyrtopleura sp.	0	0.008
Veneroida		
Cardiidae		
Laevicardium mortoni (Conrad, 1830)	0.497	0.131
Corbiculidae		
Corbicula fluminea (Müller, 1774)	23.306	33.107
Polymesoda caroliniana (Bosc, 1801)	13.281	9.052

Appendix 2. (continued)

CL OR FA Species	Dead	Live
Dreissenidae		
Mytilopsis leucophaeata (Conrad, 1831)	6.093	0.796
Lasaeidae		
Mysella planulata (Stimpson, 1851)	0.492	0.137
Lucinidae		
Anodontia alba (Link, 1807)	0.062	0
Lucina pectinata (Gmelin, 1791)	0.203	0.011
Mactridae		
Mulinia lateralis (Say, 1822)	0.923	1.734
Rangia cuneata (Sowerby, 1831)	11.418	6.619
Spisula solidissima similis (Say, 1822)	0.031	0
Pharidae		
Ensis minor (Dall, 1900)	0.031	0
Pisidiidae		
Musculium partumeium (Say, 1822)	0.031	0.011
Pisidium sp.	0.008	0
Semelidae		
Abra aequalis (Say, 1822)	0.008	0
Solecurtidae		
Tagelus plebeius (Lightfoot, 1786)	5.604	4.553
Solenidae		
Solen viridis (Say, 1821)	0.016	0
Tellinidae		
Macoma constricta (Bruguiere, 1792)	0.515	2.662
Macoma tenta (Say, 1834)	0.102	0.056
Tellina versicolor (DeKay, 1843)	0.325	2.741
Tellina sp.	1.265	0.139
Veneridae		
Anomalocardia auberiana (d'Orbigny, 1842)	1.369	0.075
Chione cancellata (Linnaeus, 1767)	2.051	0.348
Cyclinella tenuis (Recluz, 1852)	0.161	0.059
Macrocallista nimbosa (Lightfoot, 1786)	0.016	0
Mercenaria campechiensis (Gmelin, 1791)	0.130	0
Veneridae (unidentified)	0.016	0
Arcoida		
Arcidae		
Anadara transversa (Say, 1822)	0.122	0.064
Noetiidae		
Noetia ponderosa (Say, 1822)	0.016	0
Mytiloida		
Mytilidae		
Amygdalum papyrium (Conrad, 1846)	0.261	4.268
Brachidontes modiolus (Linnaeus, 1767)	0	0.127
Geukensia granosissima (Sowerby, 1914)	1.201	2.793
Ischadium recurvum (Rafinesque, 1820)	1.861	1.780
Ostreoida		
Ostreidae		
Crassostrea virginica (Gmelin, 1791)	9.923	2.626
Dendostrea frons (Linnaeus, 1758)	0.445	0
Pectinidae		
Argopecten irradians (Lamark, 1819)	0.224	0
Anomiidae		
Anomia simplex (d'Orbigny, 1842)	0.916	0
Pterioida		
Pinnidae		
Atrina serrata (Sowerby, 1825)	0.010	0
Bivalvia (unidentified)	0.062	0.317
Mollusca (unidentified)	0.016	0.023
Total	94.929	81.765

Appendix 3. Dominance of all species as a percentage of all the mean number of individuals found in each site (river or creek) sampled.

	River or Creek										
Species	Alafia	Big Slough	Blackburn	Curry	Deer Prairie	Mud	Myakka	Peace	Shakett	Shell	Weeki
Corbicula fluminea	1.23	0	0	0	4.65	0	42.12	53.32	0	0.26	1.25
Polymesoda caroliniana	19.07	40	0	1.9	44.19	21.74	17.23	3.51	2.13	46.59	21.25
Rangia cuneata	0	24	100	0	51.16	0	8.86	5.79	0	30.9	0
Tagelus plebeius	3.69	28	0	34.18	0	30.43	9.54	1.36	24.63	19.31	23.75
Crassostrea virginica	21.88	0	0	5.7	0	26.09	0	1.06	27.59	0	25
Geukensia granosissima	29.44	0	0	0	0	0	6.22	0.22	0	0	0
Amygdalum papyrium	1.23	0	0	0	0	0	0	8.28	0	0	0
Neritina usnea	5.89	8	0	0	0	0	0.45	4.95	1.31	0.77	0
Ischadium recurvum	0	0	0	1.9	0	0	0.45	2.52	16.26	1.02	15
Littoraria irrorata	4.53	0	0	1.27	0	8.69	7.92	0.47	2.46	0.51	8.75
Macoma constricta	0	0	0	0	0	13.04	0	5.16	0	0	0
Chione cancellata	0	0	0	27.85	0	0	0	0	6.9	0	0
Tellina versicolor	0	0	0	0	0	0	0	5.42	0	0	0
Mulinia lateralis	1.71	0	0	3.8	0	0	2.49	2.44	0	0.13	0
Nassarius vibex	0	0	0	3.8	0	0	0.11	2.63	0.99	0	0
Mytilopsis leucophaeata	3.56	0	0	0	0	0	3.85	0	0	0.51	0
Haminoea succinea	0	0	0	0	0	0	0	2.1	0	0	0
Laevicardium mortoni	0	0	0	10.76	0	0	0	0	2.46	0	0
Tellina sp.	0	0	0	1.27	0	0	0	0	6.9	0	2.5
Bivalvia (unidentified)	4.35	0	0	0	0	0	0	0.1	0	0	0
Anomalocardia auberiana	0	0	0	1.27	0	0	0	0	3.94	0	0
Anadara transversa	0	0	0	3.8	0	0	0	0.06	0	0	0
Melongena corona	0	0	0	0	0	0	0	0.27	0	0	2.5
Mysella planulata	2.24	0	0	0	0	0	0	0	0	0	0
Cyclinella tenuis	0	0	0	1.27	0	0	0.11	0	1.97	0	0
Macoma tenta	0.66	0	0	0	0	0	0	0	0.99	0	0
Brachidontes modiolus	0	0	0	0	0	0	0	0.25	0	0	0
Lucina pectinata	0	0	0	1.27	0	0	0	0	0	0	0
Mollusca (unidentified)	0	0	0	0	0	0	0	0.01	0.99	0	0
Planorbidae (unidentified)	0.53	0	0	0	0	0	0	0	0	0	0
Polinices duplicatus	0	0	0	0	0	0	0.11	0.06	0	0	0
Cyrtopleura sp.	0	0	0	0	0	0	0	0	0.49	0	0
Musculium partumeium	0	0	0	0	0	0	0.08	0	0	0	0

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RESEARCH NOTE

Giant African snail, *Achatina fulica*, as a snail predator

Wallace M. Meyer III[1, 2], Kenneth A. Hayes[1, 2], and Amanda L. Meyer[2]

[1] Center for Conservation Research and Training, University of Hawaii at Manoa, 3050 Maile Way, Gilmore 408, Honolulu, Hawaii 96822, U.S.A., meyerwal@hawaii.edu

[2] Department of Zoology, University of Hawaii at Manoa, 2538 McCarthy Mall, Edmonson 152, Honolulu, Hawaii 96822, U.S.A.

Abstract: Individuals of *Achatina fulica* (Bowdich, 1822) were observed preying on veronicellid slugs at two sites on the island of Oahu, Hawaii. As such, the presence of *A. fulica* may pose a greater threat to terrestrial mollusc conservation than previously imagined. It is our hope that this note provides some impetus for other researchers to explore the possible predation impacts of introduced populations of *A. fulica* and to consider the possibility that other introduced snails and slugs may be having as yet unforeseen or unnoticed impacts.

Key words: predation, introduced, invasive, alien species

Invasive species are recognized globally as a major threat to biodiversity and ecosystem health (Carlton and Geller 1993, Lydeard *et al.* 2004, Pimentel *et al.* 2005). Numerous non-native plants and animals have been implicated in the extirpation of native taxa (Vitousek *et al.* 1997, Gurevitch and Padilla 2004). However, it is difficult to assign causality to one factor such as invasive species and ignore others, *e.g.*, habitat modification and climate change (Gurevitch and Padilla 2004). Furthermore, knowledge of life histories and behavioral characteristics of alien species is usually based on studies in their native range, making it difficult to predict the interactions of a particular alien species after introduction to a new region. As such, many unpredicted interactions may occur and possibly go unnoticed. Here we report on one such interaction involving the giant African snail, *Achatina fulica* (Bowdich, 1822), in Hawaii that has gone unreported for more than half a century. We also comment on the possible implications of this interaction with native species in Hawaii as well as in other parts of the world where *A. fulica* has been introduced.

Achatina fulica is one of the largest land snails in the world, reaching up to 19 cm in length (Peterson 1957). In part because of its polyphagous diet, it has become recognized as one of the world's most damaging pests and is listed in the Global Invasive Species Database (http://www.issg.org/database/welcome/) among "One hundred of the world's worst invasive alien species" (Lowe *et al.* 2000). In addition, this snail is known as a vector of at least two human disease agents: the rat lung-worm *Parastrongylus* (=*Angiostrongylus*) *cantonensis* (Chen 1935) and a gram-negative bacterium, *Aeromonas hydrophila*, which causes a wide range of symptoms (Mead 1956, 1961, Wallace and Rosen 1969, Dean *et al.* 1970, Mead and Palcy 1992). Because of the snail's prominence as a pest and disease vector, a number of researchers have investigated its feeding and behavioral ecology in both the field and laboratory (Mead 1961, Pawson and Chase 1984, Tomiyama 1994). In addition to the 500 plant species that *A. fulica* is known to eat, the snail will also consume decaying and rotting vegetation, dung, garbage, wet paper and cardboard, dead animals, and crushed (*i.e.*, already dead) snails of its own kind (Srivastava 1992). Hence, it is surprising that no one has reported carnivorous behavior by this species in which it attacks, subdues, and consumes live prey. This note reports three instances of this predatory behavior on the island of Oahu, Hawaii. These observations are noteworthy because they indicate an alternative feeding mode for *A. fulica*, suggesting its potential to impact other species through predation.

Individuals of *Achatina fulica* were observed consuming veronicellid slugs (*Veronicella cubensis* (Pfeiffer, 1840)) at two sites on the island of Oahu (Fig. 1). Both sites were anthropogenically altered, in close proximity to housing and dominated by low shrubs. The first two observations were made in Kaneohe (21°25′02″N, 157°48′51″W) in November and December 2004. In both instances, an individual *A. fulica* (*ca.* 5 cm in shell length) was observed consuming a similar sized slug. No detailed observations were made during these first two observations.

Slug consumption by *Achatina fulica* was also observed in Hawaii Kai (21°16′12″N, 157°44′84″W) in September 2005. However, on this occasion, three smaller (10 to 15 mm in shell length) *A. fulica* were observed consuming one veronicellid slug (>5 cm in length) at the same time. In order to determine if *A. fulica* kills the slugs or whether it

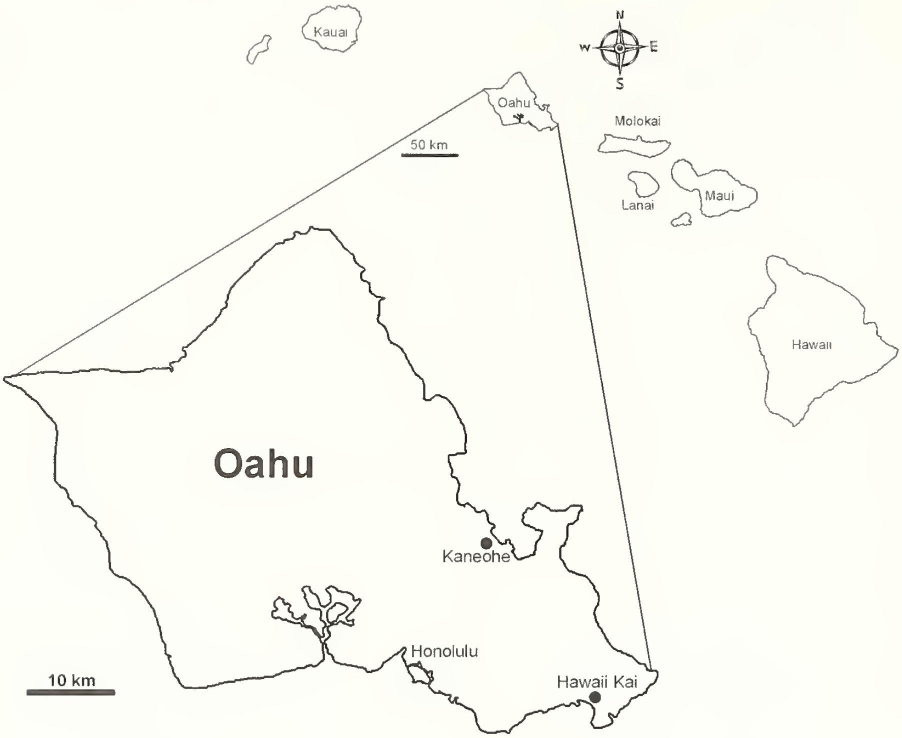

Figure 1. The two locations on Oahu (Kaneohe and Hawaii Kai) where *Achatina fulica* were observed consuming veronicellid slugs.

simply eats slugs that are already dead, a new live slug (2-3 cm in length) was collected and offered to the same three snails. It was quickly attacked by the three snails (Fig. 2). All three *A. fulica* climbed on top of the slug and proceeded to consume the integument of the slug. It took over five minutes for the snails to kill the slug. During the first three minutes, the slug crawled and pulled the snails with it as it moved. In the last two minutes, the slug seemed distressed and tried to curl up. After the slug stopped moving, the snails continued to consume the slug for a few minutes. The remainder of the slug (mostly the integument) was left in the glass dish for one day after the attack to confirm that it was actually dead.

In many low lying areas around Oahu, *Achatina fulica* and veronicellid slugs occur sympatrically. The fact that these observations have been made at different locations on the island suggests that predation by *A. fulica* on veronicellids may be common; yet an extensive literature search failed to find any previous description of this behavior. *Achatina fulica*, native to east Africa, has been introduced to many locations throughout the tropics and subtropics (Mead 1961, Mead and Palcy 1992). Given its anthropogenic distribution, it seems possible that this behavior might not be restricted to the Hawaiian Islands and to predation on veronicellid slugs.

As such, the presence of *Achatina fulica* may pose a greater threat to terrestrial mollusc conservation than previously imagined. This may be especially true because *A. fulica* has become established in areas that harbor a significant portion of the world's molluscan biodiversity and *A. fulica* populations in these areas can attain extremely high densities. For example, *A. fulica* was introduced to Brazil in 1988 and has now been recorded in 23 out of 26 states in

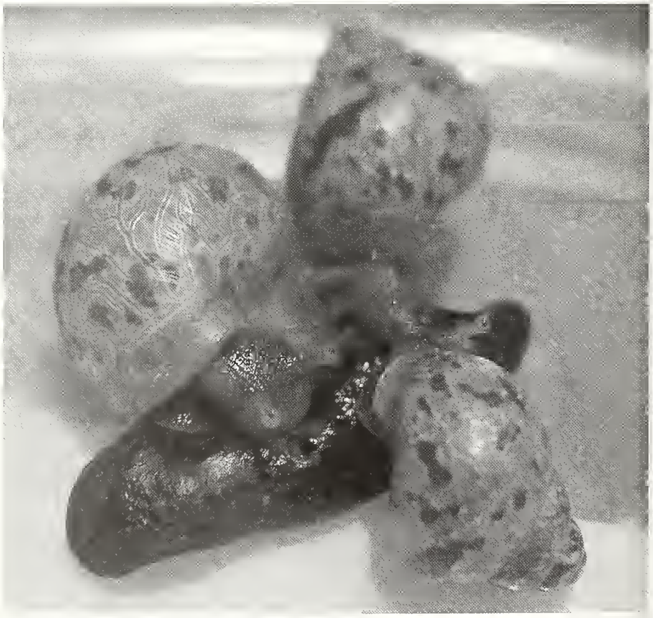

Figure 2. Three *Achatina fulica* consuming a slug that was offered after the initial observation in Hawaii Kai on September 18, 2005.

that country (Thiengo *et al.* 2007). A similar rapid spread took place after its introduction to Hawaii in the 1930s (Cowie *et al.* 1995). Both Brazil and Hawaii are known to have a wealth of land snail diversity. However, in Brazil there are many native slug species, although there are none in Hawaii (Cowie *et al.* 1995, Lewinsohn and Prado 2005). Kekauoha (1966) estimated that there were 537,600 *A. fulica* in 6.72 hectares (7.75 snails per m^2) in Hawaii, demonstrating the high densities this snail can attain in its introduced ranges. Although any statement on the impact of *A. fulica* predation on species in its newly established areas would be speculative, it seems possible that *A. fulica* could deleteriously impact the land snail fauna through competition and predation in areas where it has become established.

It is our hope that this note provides some impetus for other researchers to explore the possible predation impacts of introduced populations of *Achatina fulica* and to consider the possibility that other introduced snails and slugs may be having as yet unforeseen or unnoticed impacts. As it is difficult to make strong conclusions from only few observations, we hope that this report motivates further research. Future experiments should address: (1) what proportion of *A. fulica* have this predatory trait? (2) what proportion of the *A. fulica* diet is obtained through predation? (3) how diverse are the prey? and (4) over what geographical range does *A. fulica* display predatory behavior?

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RESEARCH NOTE

Life history and host fish identification for *Fusconaia burkei* and *Pleurobema strodeanum* (Bivalvia: Unionidae)

Megan P. White[1,*], Holly N. Blalock-Herod[2], and Paul M. Stewart[3]

[1] Department of Biological and Environmental Sciences, Troy University, Troy, Alabama 36082, U.S.A., pilamm5@wfu.edu

[2] U.S. Fish and Wildlife Service, 300 Ala Moana Blvd., Honolulu, Hawaii 96850, U.S.A., holly_herod@fws.gov

[3] Department of Biological and Environmental Sciences, Troy University, Troy, Alabama, 36082, U.S.A., mstewart@troy.edu

Abstract: We documented the period of gravidity, identified the fish host, and described the glochidia for two mussel species, *Fusconaia burkei* (Wright, 1898) and *Pleurobema strodeanum* Walker, 1922, in Eightmile Creek, Walton County, Florida. Populations of both species were checked monthly from December 2003 to October 2004 and were found to be gravid from the middle of March to late May. The size and shape of *F. burkei* and *P. strodeanum* glochidia were similar. Conglutinates released by *F. burkei* were pink-colored and cylindrical in shape, tapering sharply on both ends. *Pleurobema strodeanum* conglutinates were creamy or peach-colored and wider with a more flattened appearance than those of *F. burkei*. Ten potential host fish species were exposed to either *F. burkei* or *P. strodeanum* glochidia. We identified the blacktail shiner (*Cyprinella venusta*) as a host fish species for both *F. burkei* and *P. strodeanum*.

Key words: gravidity, glochidia, juveniles, freshwater mussels, Choctawhatchee River

North America possesses a rich freshwater mussel (Bivalvia: Unionidae) fauna, but this group is in rapid decline. Within the Conecuh-Escambia, Yellow, and Choctawhatchee River basins of Alabama and Florida, freshwater mussel populations have experienced recent declines in species richness and relative abundance (Blalock-Herod *et al.* 2005, Pilarczyk *et al.* 2006), resulting in the recent elevation of eight mussel species to candidate status under the Endangered Species Act (U.S. Fish and Wildlife Service 2004). Life history information is crucial for assessing population viability and evaluating conservation needs of this declining fauna (U.S. Fish and Wildlife Service 2003). However, host fish information has been reported for only one quarter of the mussel species in North America (Hoggarth 1992), and other life history information is also lacking for most species.

The tapered pigtoe (*Fusconaia burkei* (Wright, 1898), formerly referred to as *Quincuncina burkei* Wright, 1898) is a small mussel endemic to the Choctawhatchee River basin (Williams and Butler 1994). The fuzzy pigtoe (*Pleurobema strodeanum* Walker, 1922) is native to the Conecuh-Escambia, Yellow, and Choctawhatchee River drainages in Alabama and Florida (Clench and Turner 1956). Both of these species are candidates for protection under the Endangered Species Act, and host fish and life history information for both species are unknown. The objectives of this study were to assess the life history and host use of the tapered pigtoe and the fuzzy pigtoe in order to provide data for conservation and management decisions.

We studied both species in Eightmile Creek, Walton County, Florida, ~14.5 km east southeast of Florala, Alabama (Fig. 1). Eightmile Creek is a tributary of the Pea River (Choctawhatchee River drainage) and lies within the Southeastern Plains level III ecoregion (Griffith *et al.* 2001). At the study site, Eightmile Creek is moderately to highly sinuous, with primarily sandy substrates, a wide riparian zone, and stable, well-vegetated stream banks. Water samples from Eightmile Creek were taken monthly for 10 months. Stream water was tea-colored, as a result of natural tannins, with a mean dissolved oxygen of 7.2 mg/L ($SD = 1.6$) and mean pH of 7.0 ($SD = 0.7$). Mean turbidity was 1.9 NTU ($SD = 0.9$), and water temperature ranged from 11 to 24°C throughout the year. The creek supports a fish community of at least twenty species as well as populations of at least eight freshwater mussel species, including *Fusconaia burkei* and *Pleurobema strodeanum* (Pilarczyk *et al.* 2006).

Eightmile Creek was checked monthly from December 2003 to October 2004 for gravid mussels, except June 2004 because of high water levels. On each date, we tagged all *Fusconaia burkei* or *Pleurobema strodeanum* using numbered Floy® shellfish tags. Voucher specimens of both species were collected, preserved in 70% ethanol, and deposited in the Troy University collection.

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[*] Current Address: Department of Biology, Wake Forest University, Winston-Salem, North Carolina 27106, U.S.A.

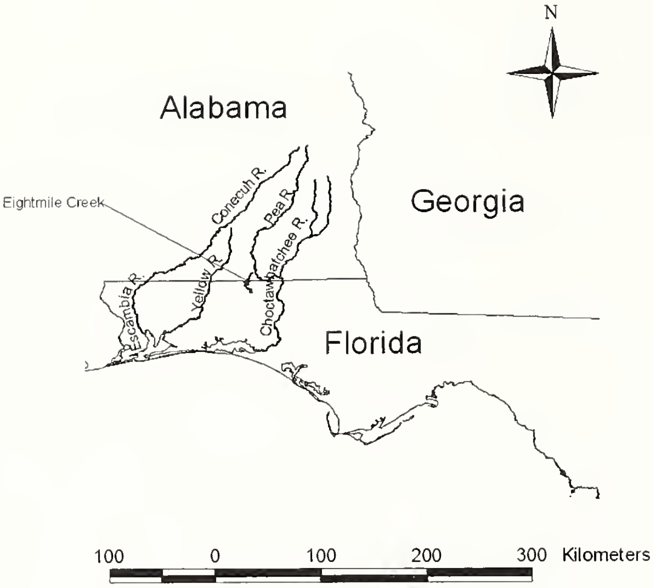

Figure 1. Study site at Eightmile Creek, Walton County, Florida.

Mussels were checked for the presence of embryos by carefully prying open the shell to inspect the gills for any inflation or color change that would indicate a gravid female. Some gravid females were transported to the laboratory to release glochidia. These mussels were individually bagged in plastic sandwich bags with stream water and transported in coolers to reduce stress and abortion of glochidia. Mussels were held at room temperature (about 22°C) in individual beakers covered with 105-µm mesh screening in an aerated aquarium containing filtered water from the study site. After glochidia were expelled, the adult mussels were returned to the collection site at Eightmile Creek.

We collected fishes for host trials using a backpack electrofisher in streams within the same watershed as Eightmile Creek where no *Fusconaia burkei* or *Pleurobema strodeanum* were present to reduce the likelihood that the fish had acquired immunity via a previous infection to the glochidia of these two species (Arey 1923, Rogers and Dimock 2003). All fishes used in host trials have been found in Eightmile Creek, with the exception of the blacktip shiner (*Lythrurus atrapiculus*), a cyprinid that occurs within the native range of the target mussel species (Mettee *et al.* 1996). Fish were transported to the laboratory in an aerated cooler and held in tanks for at least one to two weeks prior to conducting experiments to allow acclimation to the laboratory, and to slough off any residual glochidial infection.

Methods to infect fish with glochidia followed those described by O'Brien and Williams (2002). Once conglutinates were released in the laboratory, they were gently agitated to release the glochidia. Glochidial viability was checked by exposing a subsample of glochidia to NaCl. Glochidia were considered viable, and used in host fish trials, if more than 50% of the subsample snapped shut in response to the NaCl. Fishes from four families and six species were exposed to *Fusconaia burkei* glochidia over the course of 10 trials. Over the course of 20 trials, fishes from six families and 10 species were exposed to *Pleurobema strodeanum* glochidia.

After fishes were infected, each species was held individually in covered aerated beakers in a water bath at about 22°C. Every three days, the water from the beakers was sieved through a 105-µm mesh screen, and filtered material was examined under a microscope for juvenile mussels. Transformed juveniles were identified by foot movement and the presence of two adductor muscles, while untransformed glochidia were motionless and had only one adductor muscle (Karna and Millemann 1978). Once juveniles were found, the water was sieved daily. These daily checks continued for 10 days after the last juvenile was found (O'Brien and Williams 2002). If no juveniles were found after five weeks, the trial was ended. Host fish were considered those that successfully allowed the transformation of glochidia into juvenile mussels. Fish that did not produce juveniles were preserved after trials and examined under a microscope for evidence of encysted glochidia.

Several conglutinates from each species were preserved in 70% ethanol for description. We measured total length and maximum width of conglutinates to the nearest 0.01 mm for both species, using an ocular micrometer. We also measured valve length, height, and hinge length of glochidia following Hoggarth (1999). Length was measured as the maximum distance from anterior to posterior margins, and measurements were made parallel to the hinge. Height was measured as the maximum distance from dorsal to ventral margins, and measurements were made perpendicular to

Table 1. Percent gravid mussels for *Fusconaia burkei* and *Pleurobema strodeanum* out of total number of mussels of each species checked at Eightmile Creek, Florida (*, very high water level).

Date	Water temperature (°C)	*Fusconaia burkei* %	(*N*)	*Pleurobema strodeanum* %	(*N*)
12/18/2003	not recorded	0.0	(8)	0.0	(21)
1/27/2004	13.4	0.0	(5)	0.0	(21)
2/10/2004*	11.0	—	(0)	0.0	(4)
3/16/2004	17.7	25.0	(4)	53.3	(30)
3/30/2004	17.3	66.7	(9)	22.0	(41)
4/21/2004	18.3	0.0	(3)	23.8	(42)
5/27/2004	20.8	13.6	(22)	26.8	(97)
7/26/2004	23.6	0.0	(4)	0.0	(19)
8/27/2004	24.1	0.0	(8)	0.0	(24)
10/19/2004	19.6	0.0	(6)	0.0	(22)

Table 2. Fish species used, number of individuals used, trial duration (days), reason trial was ended (J, ten days after the last juvenile was detected; T, trial terminated after no juveniles were detected for five weeks; X, all fish died), and number of juveniles produced for each of the ten host fish trials performed for *Fusconaia burkei* and *Pleurobema strodeanum.*

Fish families and species	Individuals	Duration (days)	Reason trial ended	Juveniles
Fusconaia burkei				
Centrarchidae				
Lepomis macrochirus	2	37	T	0
Lepomis macrochirus	3	10	X	0
Cyprinidae				
Cyprinella venusta	4	19	X	0
Cyprinella venusta	3	32	J	35
Notropis texanus	3	35	T	0
Notropis texanus	3	8	X	0
Pteronotropis hypselopterus	1	12	X	0
Fundulidae				
Fundulus olivaceus	3	37	T	0
Fundulus olivaceus	3	34	T	0
Ictaluridae				
Noturus leptacanthus	1	8	X	0
Pleurobema strodeanum				
Aphredoderidae				
Aphredoderus sayanus	1	36	T	0
Centrarchidae				
Lepomis macrochirus	3	7	X	0
Cyprinidae				
Cyprinella venusta	3	6	X	0
Cyprinella venusta	1	27	J	84
Cyprinella venusta	1	13	X	17
Cyprinella venusta	1	16	X	71
Cyprinella venusta	1	29	J	56
Lythrurus atrapiculus	2	3	X	0
Notropis texanus	3	28	X	0
Notropis texanus	3	12	X	0
Notropis texanus	4	6	X	0
Notropis texanus	3	2	X	0
Pteronotropis hypselopterus	1	34	T	0
Fundulidae				
Fundulus olivaceus	3	4	X	0
Fundulus olivaceus	3	12	X	0
Ictaluridae				
Noturus leptacanthus	2	34	T	0
Noturus leptacanthus	1	36	T	0
Percidae				
Etheostoma edwini	1	34	T	0
Percina nigrofasciata	2	10	X	0
Percina nigrofasciata	1	11	X	0

length. Hinge length was measured in a straight line from the points at which the dorsal margins intersect the anterior and posterior margins. Student's t-tests were used to compare valve length, height, and hinge length between the two species. Other characteristics we examined were valve shape and hook presence or absence.

We tagged and examined a total of 32 *Fusconaia burkei* and 161 *Pleurobema strodeanum.* Gravid specimens of both species were found at Eightmile Creek between March 16 and May 27, 2004, indicating these species are short-term brooders, or tachytictic (Table 1). The peak period of gravidity was late March for *F. burkei* and mid-March for *P. strodeanum* (Table 1). Mean water temperature during the period in which female mussels were gravid was 18.0°C (range: 15.8-20.8°C). There was no evidence that females of either species produced multiple clutches in a year. Both species often released conglutinates within the first 24 hours following the gravidity check, indicating that handling may result in premature abortion of glochidia. While many glochidia were viable, unnecessary evaluations of gravidity should be avoided to reduce the likelihood of abortion, especially for candidate, threatened, or endangered species.

Both the inner and outer demibranchs of gravid *Fusconaia burkei* were slightly inflated and typically had a pinkish color. In the laboratory, nine *F. burkei* released pink-colored conglutinates from March 31 to April 12, 2004. Five of the nine individuals released conglutinates on only one occasion. However, the other four *F. burkei* had two to four conglutinate releases over the course of two to eight days.

Only the outer demibranchs of the gills of gravid *Pleurobema strodeanum* were inflated and had a creamy-orange color. Twenty-three *P. strodeanum* released cream-colored conglutinates in the laboratory from March 19 to April 29, 2004. The number of conglutinate releases per *P. strodeanum* individual ranged from one to four, with about half of the individuals releasing conglutinates only once. Eleven *P. strodeanum* had two to four conglutinate releases over the course of three to 12 days.

Fusconaia burkei released slender, pink-colored conglutinates that were cylindrical in shape and tapered to only one or two glochidia on both ends. *Fusconaia burkei* conglutinates measured about 1 × 6.5 mm. Conglutinates released by *Pleurobema strodeanum* were creamy or peach-colored, had a more flattened appearance, and were much wider than those of *F. burkei*. *Pleurobema strodeanum* conglutinates were also tapered, though not as narrowly as those of *F. burkei*, at both ends. *Pleurobema strodeanum* conglutinates were about 2 × 8 mm.

The size and shape of both species' glochidia were similar and not easily distinguishable. The mean valve height for *Fusconaia burkei* glochidia was 160.1 µm ($SD = 6.8$, $n = 5$), mean valve length was 167.3 µm ($SD = 4.3$, $n = 5$), and mean hinge length was 130.6 µm ($SD = 5.8$, $n = 5$). For *Pleurobema strodeanum* glochidia, mean valve height was 166.3 µm ($SD = 2.8$, $n = 5$), mean valve length was 176.5 µm ($SD = 2.8$, $n = 5$), and mean hinge length was 126.5 µm ($SD = 2.3$, $n = 5$). While there was no statistically significant difference in valve height (Student's *t*-test, $t = -1.85$, $P = 0.10$) and hinge length ($t = 1.46$, $P = 0.18$) between the two species, the valve length of *P. strodeanum* glochidia was significantly greater than that of *F. burkei* ($t = -4.02$, $P = 0.004$). The glochidial hook was absent in both *F. burkei* and *P. strodeanum*, and both species had a subelliptical valve shape.

Of the 10 host fish trials performed for *Fusconaia burkei*, only one produced juveniles (Table 2). Twenty-one days after exposure to glochidia, juveniles were found in the sieved water of *Cyprinella venusta*. This trial, the second attempt to infect *C. venusta* with *F. burkei* glochidia, resulted in the transformation of 35 glochidia into juveniles. Some juveniles were kept alive in the laboratory for at least six weeks.

Pleurobema strodeanum glochidia transformed into juveniles in four of the 20 trials (Table 2), but only on *Cyprinella venusta* individuals. A mean of 14 days from date of infection elapsed until the first transformed *P. strodeanum* juvenile was collected. The first successful trial produced 84 juveniles: 50 collected on day 15, 32 on day 16, and two on day 17. The second successful trial produced 17 juveniles on day 13, but the fish subsequently died, and no additional juveniles were collected. The third trial produced 71 juveniles: 46 collected on day 14 and 25 on day 15. The fish died on day 15, and no additional juveniles were collected. The fourth successful trial yielded 56 juveniles: six collected on day 14, 46 on day 16, and four were collected on day 18. Some juveniles from these trials were kept alive in the laboratory for over nine weeks. When fishes that did not produce juveniles were preserved and later examined, no encysted glochidia were observed on the gills or fins.

The period of gravidity documented in this study for both species is supported by existing literature. Ortmann and Walker (1922) reported a gravid female *Fusconaia burkei* at the Choctawhatchee River, Alabama, in May. Gravid *Pleurobema strodeanum* have been found at the Choctawhatchee River, Florida, during April and in the Escambia River drainage in July (H. N. Blalock-Herod, unpubl. data). Gravid females of a closely related species, *Pleurobema pyriforme* (Lea, 1857), were reported from March to July at Chickasawhatchee and Kinchafoonee Creeks in the Apalachicola, Chattahoochee and Flint Rivers Drainage (O'Brien and Williams 2002).

The successful transformation of *Fusconaia burkei* and *Pleurobema strodeanum* glochidia on *Cyprinella venusta* is also consistent with the existing literature on related taxa. *Fusconaia* typically use Cyprinidae as fish hosts. Haag and Warren (2003) reported that schools of *C. venusta* repeatedly interacted with drifting conglutinates from the related taxa *Fusconaia cerina* (Conrad, 1838) and *Pleurobema decisum* (Lea, 1831). Host fish trials resulted in *Fusconaia cerina* glochidia transforming on a wide variety of cyprinid species, including *C. venusta*; *P. decisum* glochidia also transformed consistently on *C. venusta* (Haag and Warren 2003). Additional studies confirmed that cyprinids often serve as hosts for *Pleurobema* (Haag and Warren 1997, O'Brien and Williams 2002, Layzer *et al.* 2003). The data collected in the current study will be useful in determining and implementing conservation actions for the protection of these rare mussel species.

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INDEX TO VOLUME 24(1/2)129

AUTHOR INDEX

Abidli, S. 24: 79
Adamo, S. A. 24: 25
Arrington, T. D. 24: 91
Basil, J. A. 24: 3
Blalock-Herod, H. N. 24: 121
Dasch, G. A. 24: 59
Dillon, R. T., Jr. 24: 59
Estevez, E. D. 24: 101
Flannery, M. S. 24: 101
Grasso, F. W. 24: 13
Hayes, K. A. 24: 117
King, A. J. 24: 25
Lahbib, Y. 24: 79
Leite, T. S. 24: 31
Mather, J. A. 24: 1, 31, 51
Meyer, A. L. 24: 117
Meyer, W. M. III 24: 117
Montagna, P.A. 24: 101
Palmer, T.A. 24: 101
Reeves, W. K. 24: 59
Richards, D. C. 24: 91
Rypel, A. L. 24: 97
Schwabe, E. 24: 71
Sinn, D. L. 24: 65
Soucier, C. P. 24: 3
Stewart, P. M. 24: 121
Trigui El Menif, N. 24: 79
Voight, J. R. 24: 43
White, M. P. 24: 121

PRIMARY MOLLUSCAN TAXA INDEX

[first occurrence in each paper recorded, new taxa in bold]

Abdopus 24: 1
acuta, Physa 24: 61
aequalis, Abra 24: 114
alba, Anodontia 24: 114
albus, Chiton 24: 72
Ancylidae 24: 61
Anomiidae 24: 114
antipodarum, Potamopyrgus 24: 92
Arcidae 24: 114
Arcoida 24: 114
atlantica, Placiphorella 24: 74
atratum, Cerithium 24: 113
auberiana, Anomalocardia 24: 114
Basommatophora 24: 113
belgicae, Leloupia 24: 71
Benthoctopus 24: 43
bimaculatus, Octopus 24: 52, 65
bimaculoides, Octopus 24: 15, 65
Biomphalaria 24: 59
Bivalvia 24: 97, 113, 121
boreopacifica, Graneledone 24: 44
bouveti, Callochiton 24: 71
brandaris, Bolinus 24: 79
Bullidae 24: 113
burkei, Fusconaia 24: 121
burkei, Quincuncina 24: 121
Callochiton 24: 71
Calyptraeidae 24: 113
campechiensis, Mercenaria 24: 114
cancellata, Chione 24: 107
canthylus, Benthoctopus 24: 44
Cardiidae 24: 113
caroliniana, Polymesoda 24: 101
Cephalaspidea 24: 113
Cephalopoda 24: 3
cerina, Fusconaia 24: 124
Cerithiidae 24: 113
Chitonida 24: 71
Chondropleura 24: 72
cirrosa, Eledone 24: 4
Coleoidea 24: 3
Conidae 24: 113
constricta, Macoma 24: 114
Conus 24: 113
Corbiculidae 24: 107
corona, Melongena 24: 113
cubensis, Physa 24: 61
cubensis, Veronicella 24: 117
cuneata, Rangia 24: 101
cyanea, Octopus 24: 36, 65
Cyrtopleura 24: 113
decisum, Pleurobema 24: 124
defilippi, Octopus 24: 32
dofleini, Enteroctopus 24: 1, 25, 53, 65
dorsuosus, Stenosemus 24: 74
Dreissenidae 24: 114
duplicatus, Polinices 24: 113
duryi, Heliosoma 24: 61
Ellobium 24: 113
Enteroctopus 24: 1
Epitoniidae 24: 113
erinacea, Ocenebra 24: 86
Eupleura 24: 113
exaratus, Stenosemus 24: 71
fluminea, Corbicula 24: 101
Fluminicola 24: 92
fornicata, Crepidula 24: 113
frons, Dendostrea 24: 114
fulica, Achatina 24: 117
Fusconaia 24: 124
Gastropoda 24: 59, 113
gaussae, Callochiton 24: 71
glabrata, Biomphalaria 24: 59
Graneledone 24: 43
granifera, Thiara 24: 59
granosissima, Geukensia 24: 106
Gundlachia 24: 59
haemastoma, Stramonita 24: 111
Haminoeidae 24: 113
Hapalochlaena 24: 1
Helisoma 24: 59
hummelincki, Octopus 24: 32
Hydrobiidae 24: 91
hydrothermalis, Vulcanoctopus 24: 44
idahoensis, Pyrgulopsis 24: 92
insularis, Octopus 24: 31

irradians, Argopecten 24: 114
irrorata, Littoraria 24: 101
Ischnochitonidae 24: 71
joubini, Octopus 24: 65
kerguelensis, Leptochiton 24: 71
kuhniana, Biomphalaria 24: 59
Lampsilis 24: 97
Lanx 24: 92
lapillus, Nucella 24: 86
Lasaeidae 24: 114
lateralis, Mulinia 24: 101
leucophaeata, Mytilopsis 24: 114
Littorinidae 24: 113
Loligo 24: 1
Lucinidae 24: 114
macropus, Callistoctopus 24: 32
macropus, Octopus 24: 65
Mactridae 24: 114
maculosa, Haplochlaena 24: 36
marmorata, Physa 24: 59
Melampus 24: 113
melanura, Triphora 24: 113
Melongenidae 24: 113
minor, Ensis 24: 114
mirandus, Nuttallochiton 24: 71
modiolus, Brachidontes 24: 114
mortoni, Laevicardium 24: 113
Muricidae 24: 79, 113
Myoida 24: 113
Mytilidae 24: 106
Mytiloida 24: 114
Nassariidae 24: 113
Naticidae 24: 113
natricina, Physa 24: 92
Nautilus 24: 1, 4
Neogastropoda 24: 113
Neoloricata 24: 71
Neotaenioglossa 24: 113
Neritidae 24: 113
Neritopsina 24: 113
nimbosa, Macrocallista 24: 114
Noetiidae 24: 114
Octopodidae 24: 32, 43
Octopus 24: 1, 13, 68
officinalis, Sepia 24: 9, 25, 31, 51
opalescens, Loligo 24: 36
Ostreidae 24: 114
Ostreoida 24: 114
papyrium, Amygdalum 24: 106
partumeium, Musculium 24: 114
pealeii, Loligo 24: 15, 25
pectinata, Lucina 24: 114
Pectinidae 24: 114
Pharidae 24: 114
Pholadidae 24: 113
Physa 24: 59
Pinnidae 24: 114
Pisidiidae 24: 114
Pisidium 24: 114
Placiphorella 24: 74
Planorbidae 24: 113
planulata, Mysella 24: 114
plebeius, Tagelus 24: 101
Pleurobema 24: 124
Polyplacophora 24: 71
pompilius, Nautilus 24: 3
ponderosa, Noetia 24: 114
Pterioida 24: 114
Pulmonata 24: 113
punctulata, Neritina 24: 59
pyriforme, Pleurobema 24: 124
radiata, Gundlachia 24: 59
recurvum, Ischadium 24: 101
reticulata, Hinia 24: 79
robusta, Pyrgulopsis 24: 92
rubescens, Octopus 24: 37, 52
rupicola, Epitonium 24: 113
scutulata, Littorina 24: 66
Semelidae 24: 114
Sepia 24: 1
serpenticola, Taylorconcha 24: 91
serrata, Artina 24: 114
setulosum, Hemiathrum 24: 71
simplex, Anomia 24: 114
simplicissimus, Ischnochiton 24: 72
simplicissimus, Stenosemus 24: 71
Solecurtidae 24: 114
Solenidae 24: 114
solidissima, Spisula 24: 114
Stenosemus 24: 72
straminea, Biomphalaria 24: 59
striata, Bulla 24: 113
strodeanum, Pleurobema 24: 121
succinea, Haminoea 24: 113
tampaensis, Urosalpinx 24: 113
tasmanica, Euprymna 24: 52
Taylorconcha 24: 91
Tellina 24: 114
Tellinidae 24: 106
tenta, Macoma 24: 114
tenuis, Cyclinella 24: 107
teres, Lampsilis 24: 97
transversa, Anadara 24: 114
Triphoridae 24: 113
trivolvis, Helisoma 24: 59
trunculus, Hexaplex 24: 79
tuberculata, Melanoides 24: 59
undatum, Buccinum 24: 87
Unionidae 24: 121
usnea, Neritina 24: 101
utahensis, Valvata 24: 92
variabilis, Provanna 24: 45
Veneridae 24: 106
Veneroida 24: 113
versicolor, Tellina 24: 108
vibex, Nassarius 24: 113
virginica, Crassostrea 24: 101
viridis, Solen 24: 114
Vulcanoctopus 24: 43
vulgaris, Octopus 24: 1, 21, 28, 31, 53, 65
zschaui, Tonicina 24: 71

LIST OF REVIEWERS FOR 2006 AND 2007

Adamo, Shelley
Barnhart, Chris
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Shaw, Paul
Shea, Elizabeth (several articles)
Shively, Steven
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Seed, R. 1980. Shell growth and form in the Bivalvia. *In*: D. C. Rhoads and R. A. Lutz, eds., *Skeletal Growth of Aquatic Organisms*. Plenum Press, New York, New York. Pp. 23-67.

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74th Annual Meeting

American Malacological Society

Carbondale, Illinois
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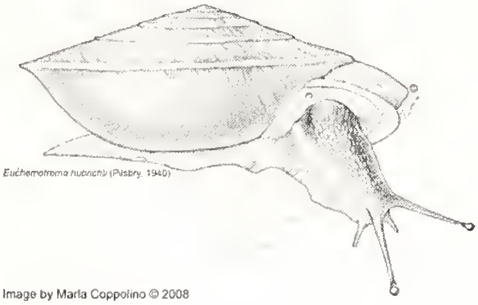

Euchemotrema hubrichti (Pilsbry, 1940)

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The American Malacological Society will hold its 74th annual meeting in Carbondale, Illinois from June 29 - July 3, 2008. The venue will be the Southern Illinois University Student Center, which houses an auditorium, several ballrooms and meeting rooms, and a number of restaurants and coffee shops. The conference will begin with an icebreaker on Sunday evening. Special events will include an outdoor reception at Blue Sky Vineyard (www.blueskyvineyard.com) on Monday night, a poster session and the AMS Auction of molluscan miscellany on Tuesday night, and a barbecue banquet (with vegetarian options) at the 17th Street Bar & Grill Warehouse, Southern Illinois' most unique banquet facility on Wednesday night. 17th Street Bar & Grill was recently recognized by Food Network and Bon Appétit as the home of the best ribs in the country.

The special sessions and symposia will include:

- a land snail conservation symposium and workshop in honor of the late Leslie Hubricht, organized by Kathryn Perez (University of North Carolina – Chapel Hill/Duke University), Jay Cordeiro (NatureServe), Jochen Gerber (Field Museum of Natural History) and Kevin Roe (Iowa State University)

- a symposium on molluscan taxonomy in the 21st century, organized by Benoît Dayrat (UC Merced)

- a special session on cephalopod biology organized by Frank Anderson, Christine Huffard (Monterey Bay Aquarium Research Institute), and Elizabeth Shea (Delaware Museum of Natural History)

On Thursday, two field trips will introduce meeting participants to wonderful mollusk habitats in southern Illinois. Participants will be able to take a tour of the Larue Pine Hills/Otter Pond Research Natural Area, a fantastic area of limestone bluffs and outcrops (and home of *Euchemotrema hubrichti*, the conference mascot), or a trip to local aquatic habitats to search for freshwater bivalves and gastropods.

Visitors to Carbondale usually travel through either St. Louis or Chicago, though Memphis is also an option. There is a convenient shuttle service from St. Louis Lambert International Airport to Carbondale. From Chicago or Memphis, you can take a train—*The City of New Orleans*, which stops in Carbondale on its Chicago-to-New Orleans route. Flights also arrive at the Williamson County airport several times daily. The airport is located 16 miles east of the SIU campus.

For meeting registration and abstract submission information, please visit http://malacological.org/meetings/next.html. We look forward to seeing you in Carbondale, Illinois in 2008!

For more information, please contact:

Frank E. Anderson
Department of Zoology
Southern Illinois University
Carbondale, IL 62901

Phone: 618-453-4136
E-mail: feander@siu.edu

The American Malacological Society

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Symposium on the Current State of Land Snail Conservation & Land Snail Identification Workshop

The American Malacological Society is pleased to announce the *Leslie Hubricht Symposium and Workshop on the taxonomy, distribution and conservation of terrestrial Gastropods.* **The land snail symposium & workshop are aimed at AMS members, state and federal agency employees, and others who are seeking training in land snail collecting, identification, and ecology.** Two of the major goals of the symposium and associated workshop are (1) to provide an opportunity for networking among established land snail researchers as well individuals who lack taxonomic experience but are responsible for day-to-day land snail conservation and (2) to offer an opportunity for non-land snail experts to receive training in basic aspects of land snail biology and identification. Workshop attendees are invited to bring their own shells to identify!

Workshop topics covered include:

- Introduction to land snail collecting strategies
- Introduction to ID terminology and literature
- The major families of macro and micro land snails of North America
- Strategies for the conservation of invertebrates with emphasis on terrestrial snails/slugs
- Introduction to the identification of invasive snails and slugs

For more specific information on workshop registration, symposium topics, and accommodations in and around Carbondale as it develops, please visit the meeting website http://www.malacological.org/meetings/next.html.

Patterns of activity cycles in juvenile California two-spot octopuses (*Octopus bimaculoides*).
DAVID L. SINN 65

Discovery of the South African polyplacophoran *Stenosemus simplicissimus* (Thiele, 1906) (Mollusca, Polyplacophora, Ischnochitonidae) in the Southern Ocean.
ENRICO SCHWABE 71

Imposex level and penis malformation in *Hexaplex trunculus* from the Tunisian coast.
YOUSSEF LAHBIB, SAMI ABIDLI, and NAJOUA TRIGUI EL MENIF 79

Threatened Bliss Rapids snail's susceptibility to desiccation: Potential impact from hydroelectric facilities. **DAVID C. RICHARDS and TRISTAN D. ARRINGTON** 91

Field observations of the nocturnal mantle-flap lure of *Lampsilis teres*. **ANDREW LEE RYPEL** 97

Meta-analysis of the relationship between salinity and molluscs in tidal river estuaries of southwest Florida, U.S.A. **PAUL A. MONTAGNA, ERNEST D. ESTEVEZ, TERRY A. PALMER, and MICHAEL S. FLANNERY** 101

Research Note: Giant African snail, *Achatina fulica*, as a snail predator.
WALLACE M. MEYER III, KENNETH A. HAYES, and AMANDA L. MEYER 117

Research Note: Life history and host fish identification for *Fusconaia burkei* and *Pleurobema strodeanum* (Bivalvia: Unionidae). **MEGAN P. WHITE, HOLLY N. BLALOCK-HEROD, and PAUL M. STEWART** 121

Index to Vol. 24 126

Membership Form 129

Information for Contributors 131

Meeting Announcement 133

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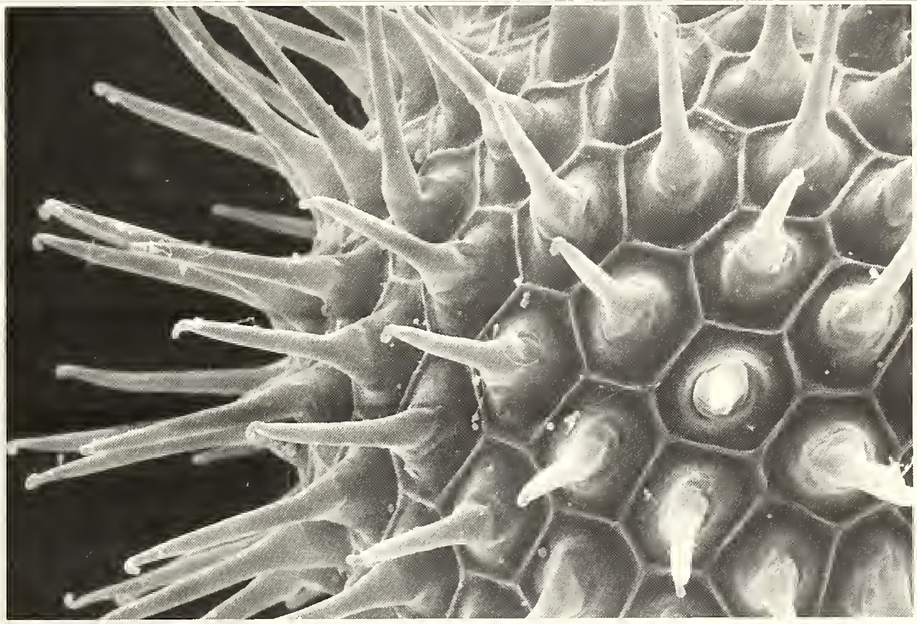
VOLUME 25 | 28 JULY 2008 | NUMBER 1/2



Status of freshwater native mussels (Unionidae) in the Oklahoma section of the Verdigris River after introduction of the zebra mussel (*Dreissena polymorpha* Pallas, 1771).
CHAD J. BOECKMAN and JOSEPH R. BIDWELL 1

Spatial distribution of soft-bottom molluscs in the Ensenada de San Simón (NW Spain).
EVA CACABELOS, PATRICIA QUINTAS, and JESÚS S. TRONCOSO 9

Introduction to the symposium "Advances in Chiton Research". **DOUGLAS J. EERNISSE** 21

New information about *Echinochiton dufoei*, the Ordovician spiny chiton.
JOHN POJETA, Jr. and JIMMIE DUFOE 25

Methods of sample preparation of radula epithelial tissue in chitons (Mollusca: Polyplacophora).
JEREMY A. SHAW, DAVID J. MACEY, PETA L. CLODE, LESLEY R. BROOKER, RICHARD I. WEBB, EDWARD J. STOCKDALE, and RACHEL M. BINKS 35

Gross anatomy and positional homology of gills, gonopores, and nephridiopores in "basal" living chitons (Polyplacophora: Lepidopleurina). **JULIA D. SIGWART** 43

Aesthete canal morphology in the Mopaliidae (Polyplacophora). **MICHAEL J. VENDRASCO, CHRISTINE Z. FERNANDEZ, DOUGLAS J. EERNISSE, and BRUCE RUNNEGAR** 51

continued on back cover

Cover photo: Fertilization in the chiton *Stenosemus albus* (Linnaeus, 1767) by Buckland-Nicks

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AMERICAN MALACOLOGICAL BULLETIN

CONTENTS

VOLUME 25 | NUMBER 1/2

Status of freshwater native mussels (Unionidae) in the Oklahoma section of the Verdigris River after introduction of the zebra mussel (*Dreissena polymorpha* Pallas, 1771). **CHAD J. BOECKMAN and JOSEPH R. BIDWELL** 1

Spatial distribution of soft-bottom molluscs in the Ensenada de San Simón (NW Spain). **EVA CACABELOS, PATRICIA QUINTAS, and JESÚS S. TRONCOSO** 9

Introduction to the symposium "Advances in Chiton Research". **DOUGLAS J. EERNISSE** 21

New information about *Echinochiton dufoei*, the Ordovician spiny chiton. **JOHN POJETA, Jr. and JIMMIE DUFOE** 25

Methods of sample preparation of radula epithelial tissue in chitons (Mollusca: Polyplacophora). **JEREMY A. SHAW, DAVID J. MACEY, PETA L. CLODE, LESLEY R. BROOKER, RICHARD I. WEBB, EDWARD J. STOCKDALE, and RACHEL M. BINKS** 35

Gross anatomy and positional homology of gills, gonopores, and nephridiopores in "basal" living chitons (Polyplacophora: Lepidopleurina). **JULIA D. SIGWART** 43

Aesthete canal morphology in the Mopaliidae (Polyplacophora). **MICHAEL J. VENDRASCO, CHRISTINE Z. FERNANDEZ, DOUGLAS J. EERNISSE, and BRUCE RUNNEGAR** 51

Mopalia kennerleyi Carpenter, 1864, a forgotten species and its southern analogue *Mopalia ciliata* (Sowerby, 1840). **ROGER N. CLARK** 71

Two new chitons of the genus *Tripoplax* Berry, 1919 from the Monterey Sea Canyon. **ROGER N. CLARK** 77

The effect of sampling bias on the fossil record of chitons (Mollusca, Polyplacophora). **STEPHANEY S. PUCHALSKI, DOUGLAS J. EERNISSE, and CLAUDIA C. JOHNSON** 87

Fertilization biology and the evolution of chitons. **JOHN BUCKLAND-NICKS** 97

Chitons (Mollusca: Polyplacophora) associated with hydrothermal vents and methane seeps around Japan, with descriptions of three new species. **HIROSHI SAITO, KATSUNORI FUJIKURA, and SHINJI TSUCHIDA** 113

Index to Vol. 25 125

Membership Form for 2008 128

Information for Contributors 130

Amer. Malac. Bull. **25**: 1-8 (2008)

Status of freshwater native mussels (Unionidae) in the Oklahoma section of the Verdigris River after introduction of the zebra mussel (*Dreissena polymorpha* Pallas, 1771)

Chad J. Boeckman and Joseph R. Bidwell

Oklahoma State University, Ecotoxicology and Water Quality Research Laboratory, 430 Life Sciences West, Stillwater, Oklahoma 74078, U.S.A., chadjb@okstate.edu

Abstract: The Verdigris River in Kansas and Oklahoma, USA once held a diverse native unionid mussel fauna although a number of these populations have declined in richness and abundance. There is recent evidence that populations of some species of unionid mussels are increasing in parts of the Verdigris River in Kansas, but no current data exist for Oklahoma. In addition, zebra mussels (*Dreissena polymorpha* Pallas, 1771) have been introduced into a major impoundment on the Verdigris River, Oologah Lake, and may further threaten mussel populations downstream from this reservoir. This study updates the distribution and abundance of native mussels in the Oklahoma portion of the Verdigris River upstream and downstream of Oologah Lake, and documents the current distribution of zebra mussels in this region. Thirty-one sites were sampled and a significant increase in species richness and abundance of native mussels was observed as compared to a 1997 study. Two species of special interest, *Cyprogenia aberti* (Conrad, 1850) and *Quadrula cylindrica* (Say, 1817), were found. Zebra mussels were not found at sites upstream of Oologah Lake but were present at every downstream site. In September 2006, zebra mussel byssal threads were observed on shells of a number of native mussels downstream from Oologah Lake, but unionid richness and abundance were not significantly different between sites above and below the reservoir.

Key words: invasive species, *Quadrula*, field survey, impoundment, semi-quantitative sampling

Freshwater mussels are considered one of the most imperiled groups of organisms in the world, with 70% of taxa within the family Unionidae considered of special concern or endangered (Bogan 1993, Strayer *et al.* 2004, Warren and Haag 2005, Jones *et al.* 2006). As for many other organisms experiencing population declines, loss of native mussels has been attributed to habitat alteration (Vaughn and Taylor 1999, Garner and McGregor 2001), point and non-point source pollution (Richter *et al.* 1997), and invasive species (Burlakova *et al.* 2000, Strayer *et al.* 2004).

The Verdigris River originates in the Flint Hills of southeastern Kansas (Schuster 1979) and flows south into Oklahoma where it joins the Arkansas River near Muskogee, Oklahoma. Historically, the Oklahoma portion of the Verdigris contained a diverse assemblage of native unionid mussels, with Isely (1924) describing this region as among the richest mussel faunas in the state. Since that time, significant portions of the river have been altered by impoundments and the lowest reaches have been dredged to create a navigation channel to the Arkansas River (USACE 2007). Agricultural activity and urban and industrial development have led to pollutant and sediment input, degrading water quality. Surveys conducted in both Kansas and Oklahoma in the 1990s indicated an overall decline in Verdigris River mussel populations compared to earlier studies (Obermeyer *et al.* 1997a, Vaughn 1998).

Interestingly, more recent surveys in Kansas have reported increases in the densities of some unionid taxa. For example, Miller and Lynott (2006) report increases in the abundance of 10 mussel species in the Kansas portion of the Verdigris between 1991 and 2003, a change they attributed to improved habitat quality. The last mussel survey conducted in the Oklahoma portion of the Verdigris was by Vaughn (1998). In addition, zebra mussels (*Dreissena polymorpha* Pallas, 1771) were discovered in Oologah Lake, an impoundment of the Verdigris in Oklahoma, in the spring of 2003 (Laney 2005). While the distribution of *D. polymorpha* in the Verdigris River was largely unknown, the potential impact of these invasives on unionid mussels is well established (Ricciardi *et al.* 1996, Baker and Hornbach 1997, Schloesser *et al.* 2006). The objectives of this study were to survey the mussel communities in the Oklahoma portion of the Verdigris River to determine if increases similar to those in Kansas were occurring, to characterize the extent to which zebra mussels occur along this section of the Verdigris, and to gather preliminary data regarding their potential interaction with native mussels.

MATERIALS AND METHODS

Sampling locations in the Verdigris River were selected to correspond with sites previously sampled by Vaughn (1998). Sample sites were mostly riffle areas and mussel surveys were conducted in runs immediately downstream of each riffle. Substrate ranged from loose gravel to cobble. Physical-chemical characteristics (temperature, dissolved oxygen, pH, and conductivity) were recorded using a Hydrolab Quanta multi-parameter probe (Hydrolab Corporation, Austin, Texas). Additionally, river water was collected in acid-washed polyethylene bottles and transported back to the laboratory on ice for determination of titratable alkalinity and hardness (APHA 1998).

Mussel surveys involved timed snorkel searches by two individuals. Surveys were conducted for at least 15 min (2 people × 15 min = 30 min total search time), with longer time intervals devoted to locations that contained greater areas of stable gravel substrate with good flow and low levels of fine sediment. Mussels were carefully removed from the substrate and placed in mesh bags. After each survey, mussels were sorted, measured to the nearest 0.01 mm maximum length using digital calipers (Fisher Scientific, Pittsburgh, Pennsylvania), and identified to species using keys by Cummings and Mayer (1992), Oesch (1995), and Couch (1997). Mussels were then carefully returned to the river throughout the search area. Live voucher specimens were not collected due to limited representatives of some species; however, digital photographs and relict shells from each sample site were deposited at the Ecotoxicology and Water Quality Research Laboratory at Oklahoma State University, Stillwater, Oklahoma.

Comparisons of mussel richness and abundance were made using 2-sample paired *t*-test for means ($\alpha = 0.05$) performed with SigmaStat version 3.1 (Systat Software Inc., San Jose, California). Regression coefficients and elevation of richness versus log-transformed abundance curves were generated for the present study and that conducted by Vaughn (1998) and were compared using *t*-tests at $\alpha = 0.05$ (Zar 1999).

RESULTS

A total of 31 sites (20 above Oologah Lake and 11 below) were surveyed for mussels between July and October 2006. Sites above Oologah Lake started approx. 0.5 km after the Verdigris River crossed the Oklahoma-Kansas border and ended approx. 28 km above Oologah Lake. The section of river between the last upstream site and the reservoir was not surveyed because of a general lack of riverine mussel habitat as the river widens and becomes more lake-like. Sites below Oologah Lake were located 1-25 km below the Oologah Lake dam. Habitat beyond this point also was considered unsuitable due to dredging in the McClellan-Kerr Navigation Channel.

Temperature across all sites ranged between 20-35 °C, dissolved oxygen between 6.0-11.9 mg/L, pH between 6-8 standard units, alkalinity between 120-170 mg/L $CaCO_3$, and hardness between 112-156 mg/L as $CaCO_3$. Seventeen species of mussels were identified from the Verdigris River as a whole (Fig. 1), with *Quadrula metanevra* (Rafinesque, 1820) being most abundant and *Quadrula nodulata* (Rafinesque, 1820) the least. *Cyprogenia aberti* (Conrad, 1850), *Lampsilis teres* (Rafinesque, 1820), and *Lasmigona complanata* (Barnes, 1823) were found only in sites above Oologah Lake, while *Megalonaias nervosa* (Rafinesque, 1820), *Quadrula cylindrica* (Say, 1817), and *Q. nodulata* were found only at sites below the lake. Generally, abundant species were also the most widely distributed except for *Q. cylindrica* which was found only at three locations but was the sixth most abundant species (Fig. 2). Size-frequency distributions were developed

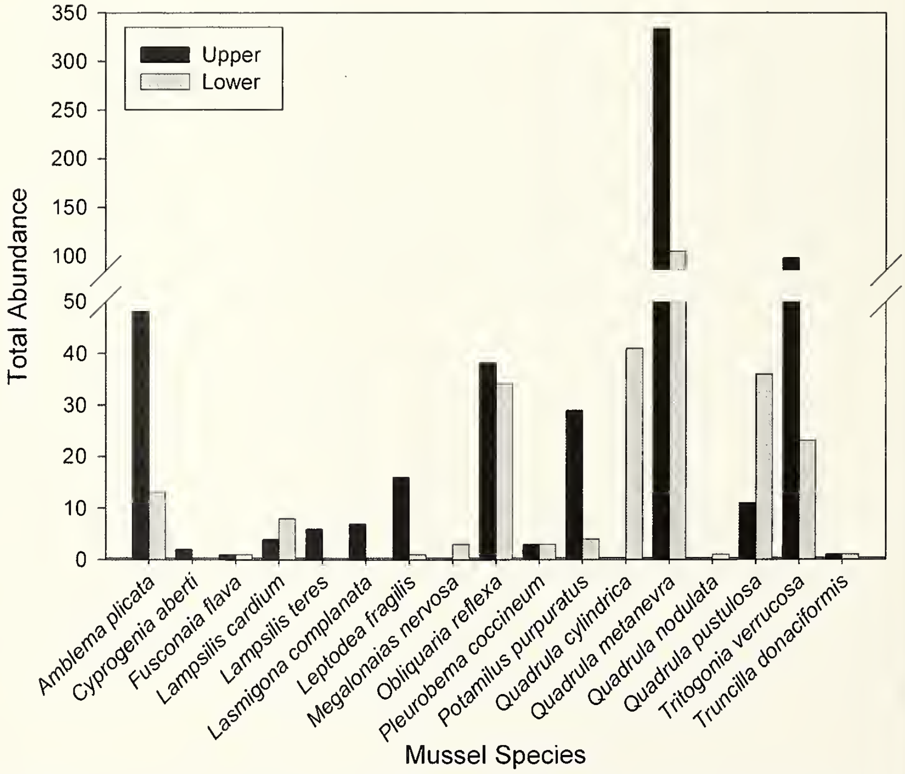

Figure 1. Abundance of native mussels (total number found) in the Verdigris River, Oklahoma, above (Upper) and below (Lower) Oologah Lake.

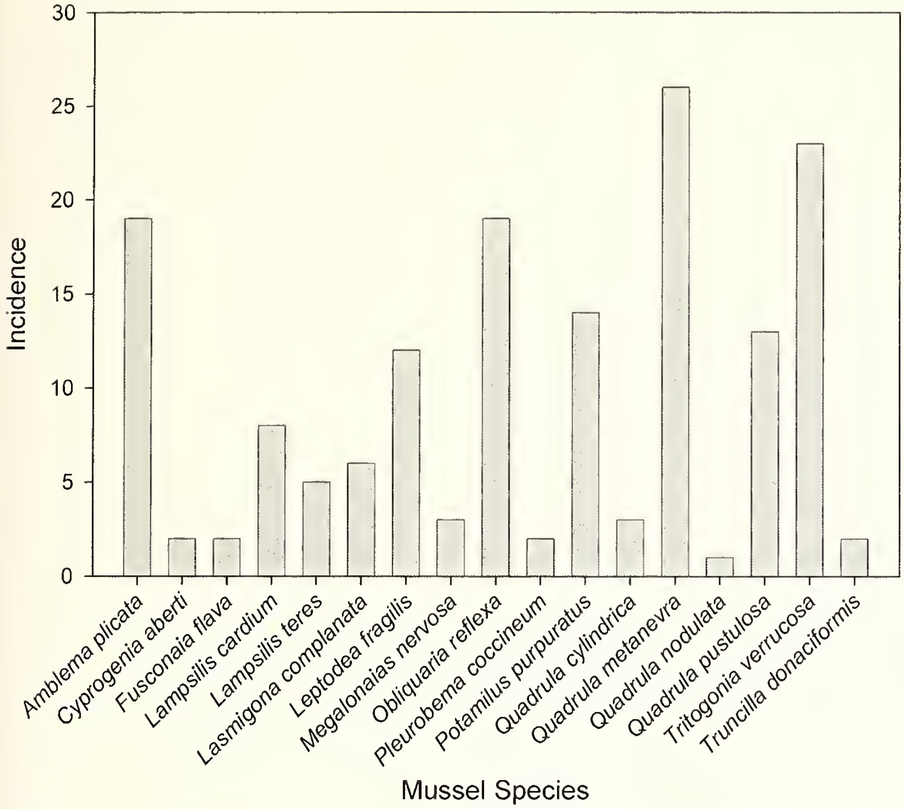

Figure 2. Incidence (number of sites at which a species occurred) of native mussels for the Verdigris River, Oklahoma. Maximum incidence = 31.

Table 1. Mean richness and abundance (#/h, mussels found per hour sampling effort) for sites within the Verdigris River, Oklahoma. Upper, sites above Oologah Lake; Lower, sites below the lake; All, all sites combined.

Sites	Mean richness	Mean #/h	*N*
Upper	5.60	39.31	20
Lower	4.36	35.66	11
All	5.16	38.01	31

for the four most abundant species, *Q. metanevra*, *Tritogonia verrucosa* (Rafinesque, 1820), *Obiquaria reflexa* (Rafinesque, 1820), and *Amblema plicata* (Say, 1817) (Fig. 3). The distributions for all four species were negatively skewed, indicating fewer small individuals than might be predicted given a normal distribution, with kurtosis values generally positive except for *O. reflexa* with a -0.74 value indicating a slightly more platykurtic (uniform) distribution.

Live mussels were found at all but 2 sites. Timed abundance estimates for sites with live mussels ranged from 3 to 156 mussels per hour (#/h) with a mean of 38 for all 31 sites (Table 1). Taxa richness ranged from 2 to 10 species per site with a mean of 5.2 species for all 31 sites combined.

No evidence of zebra mussels was found at any site above Oologah Lake; however, zebra mussels and byssal threads were found on substrate and unionid shells at every site below the lake. While many native mussels collected below the lake had byssal threads on the valves, few had live zebra mussels attached. In order to assess the potential impact of zebra mussels on native mussel community composition, species richness and abundance were estimated separately for sites above and below the lake. Sample locations above the lake had a mean richness of 5.6 species per site, and an abundance of 39.3/h (Table 1). Sites below Oologah Lake had a mean richness of 4.4 species per site with an abundance of 35.7/h (Table 1). There was no significant difference in overall mussel species richness or abundance between upstream and downstream sites ($P = 0.12$ and $P = 0.41$, respectively).

While no differences in mussel richness and abundance were apparent when sites were combined within upstream and downstream sections, a downstream longitudinal gradient in these parameters was apparent. Both mussel abundance ($r^2 = 0.697$, $N = 11$, $P = 0.001$) and species richness ($r^2 = 0.539$, $N = 11$, $P = 0.014$) were significantly positively associated with distance from the dam (Fig. 4). However, these analyses may be influenced by two downstream sites that had the greatest abundance of any of the sample locations. When these two sites are removed from the analyses, the longitudinal relationship is no longer significant (#/h: $r^2 = 0.31$, $N = 9$, $P = 0.120$; richness: $r^2 = 0.38$, $N = 9$, $P = 0.077$).

DISCUSSION

Sampling locations in the Verdigris River corresponded to a previous study (Vaughn 1998) to reassess the status of the native mussel community since the discovery of zebra mussels in Oologah Lake in June 2003. Vaughn (1998) identified 16 species of mussels, compared with 17 in this study.

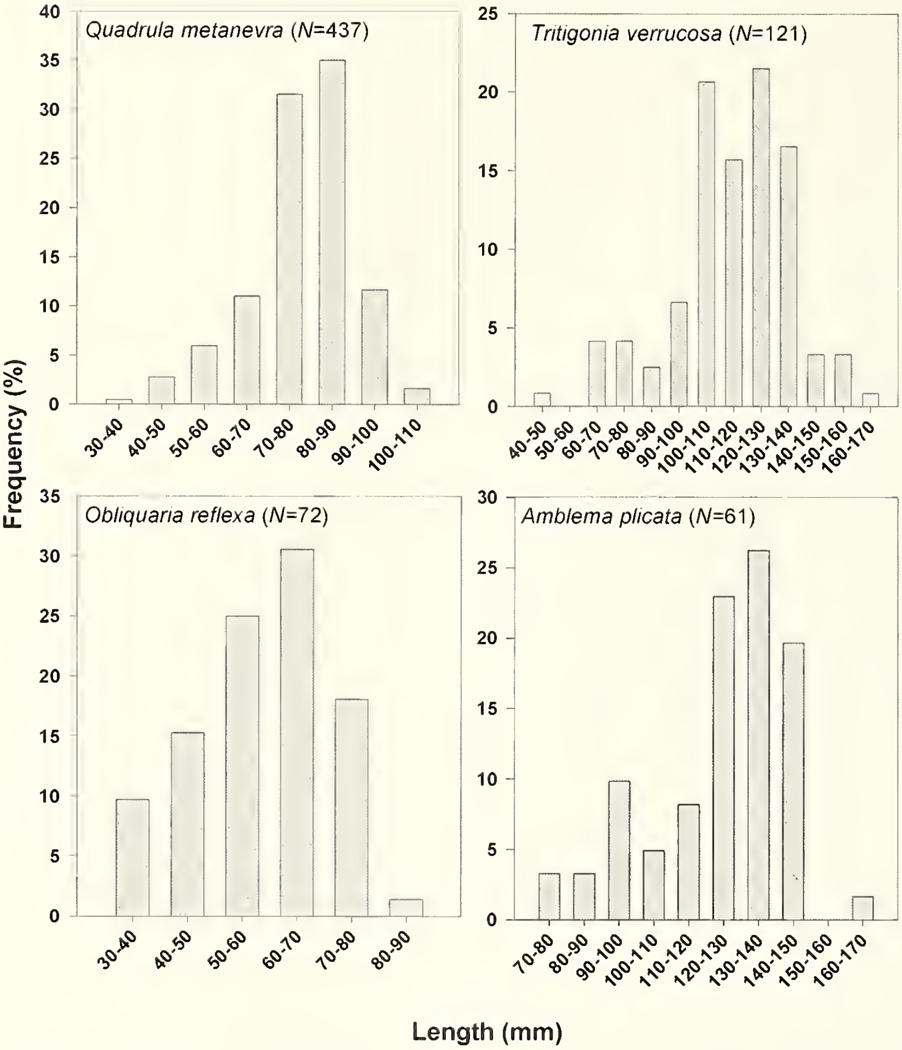

Figure 3. Size-frequency distributions for the four most abundant mussel species in the Verdigris River, Oklahoma. *N*, number of observations for that species.

While species composition was largely similar between the two surveys, *Potamilus ohiensis* (Rafinesque, 1820) and *Quadrula quadrula* (Rafinesque, 1820) were not found in 2006. In contrast, live *Cyprogenia aberti*, *Quadrula cylindrica*, and *Quadrula nodulata* were found in the 2006 survey while only relict shells of these species were found in the previous survey. Of these, *C. aberti* and *Q. cylindrica* are of particular interest because *C. aberti* was once thought extirpated from the state (Mather 1990, Serb 2006), and *Q. cylindrica* is a federal candidate for listing as an endangered species (David Martinez, pers. comm., U. S. Fish and Wildlife Service). Only 2 *C. aberti* were found, and both individuals were from sites near the Oklahoma-Kansas border. As abundance of this species has increased in the Kansas portion of the Verdigris (Miller and Lynott 2006), these upstream populations may be responsible for the reintroduction into Oklahoma through mechanisms such as fish host dispersal or downstream transport of juveniles after detaching from the fish host (Morales *et al.* 2006).

While *Quadrula cylindrica* was the sixth most abundant species, it was found only in a short river section downstream from Oologah Lake, an area also infested with zebra mussels. Lee *et al.* (1998) and Berg *et al.* (2007) suggest host-fish vagility may explain unionid distribution patterns, with mussels that utilize fish hosts with greater home ranges typically showing greater abundance and distribution. The known fish hosts for *Q. cylindrica* include several species of *Notropis* (Yeager and Neves 1986), which have relatively small home ranges (Goforth and Foltz 1998). This characteristic of its fish host may explain the limited distribution of *Q. cylindrica* in the Verdigris.

Size-frequency distributions of the 4 most commonly encountered mussel species did not include individuals less than 30 mm in length (70 mm for *Amblema plicata*) although this may have been a sampling artifact as timed snorkel searches bias against encountering very small or particularly cryptic individuals (Hornbach and Deneka 1996, Obermeyer 1998, Metcalfe-Smith *et al.* 2000). Furthermore, juveniles frequently bury in the substrate, making detection difficult using snorkel searches (Neves and Widlak 1987, Amyot and Downing 1991, Yeager *et al.* 1994, Sparks and Strayer 1998). Timed snorkel search techniques are, however, more commonly used for determining mussel richness and locating rare species (Metcalfe-Smith *et al.* 2000, Vaughn and Spooner 2004) and are more cost effective when surveys of large areas are needed as compared with quadrat methods.

Vaughn (1998) reported a mean abundance for all 31 sites of 14.4 mussels/h and a mean richness of 3.3 species per site. Current abundance and richness across all 31 sites were 38.0/h and 5.2 species per site, significantly greater than reported by Vaughn ($P = 0.002$ and $P = 0.001$, respectively). Mean sampling time between the two surveys was not significantly different ($P = 0.65$) with 46.8 min in this survey vs.

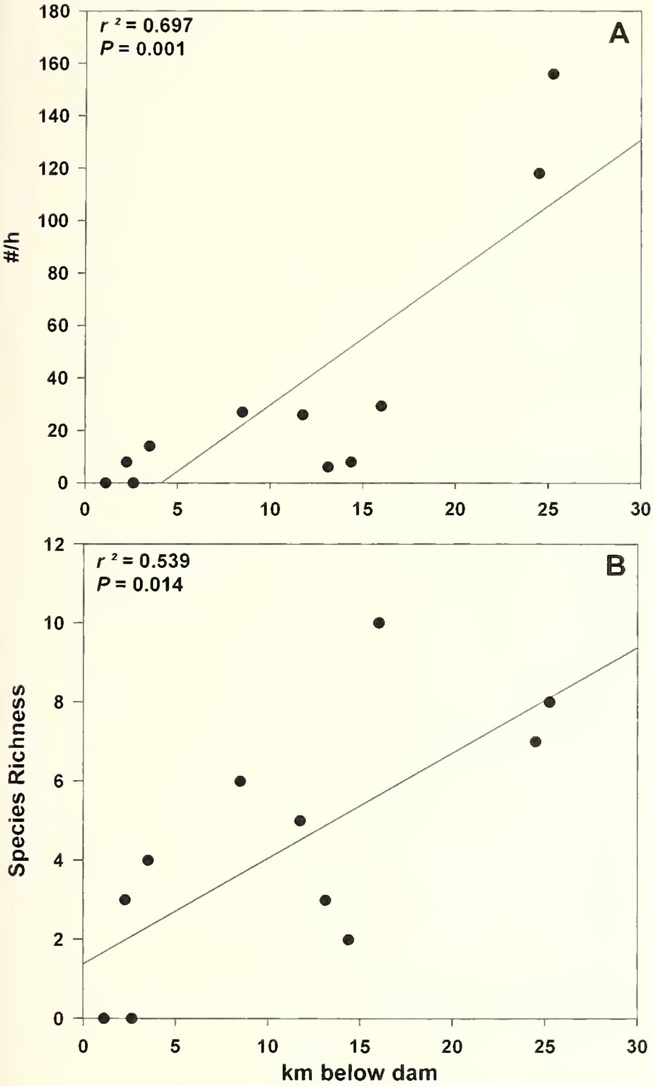

Figure 4. A, Distance from the dam vs. mussel abundance (#/h). B, richness in Verdigris River, Oklahoma for sites below Lake Oologah dam. Coefficients of determination and significance levels are shown.

49.8 min in Vaughn (1998). To evaluate the extent to which the increase in richness resulted from locating more mussels overall, abundance vs. richness curves were prepared from both studies (Fig. 5). These curves were linearized by log-transforming abundance to facilitate statistical analysis. There was no significant difference between studies in the regression coefficient (36.7 for the present study vs. 65.7 for the Vaughn study, $P > 0.05$) or elevation ($P > 0.05$), indicating that mussel abundance explained a similar degree of variation in taxonomic richness in both studies. Thus the greater taxa richness in the present study appears to be explained by our finding a greater number of mussels during the timed searches.

Given the semi-quantitative sampling employed in both surveys, care should be taken not to over-interpret these data. Strayer (1999) found low statistical power for detecting population declines when using presence/absence techniques; therefore, these results should at a minimum support the need for more quantitative techniques throughout the study area. Miller and Obermeyer (1997) and Miller and Lynott (2006) reported an increase in 10 different species in the Kansas portion of the Verdigris River relative to 1991 levels, using quadrat methods. They attribute this increase to improvements in habitat quality, namely reduction in pollution, increase in fish hosts, and lack of severe drought. These same factors may be working to improve native mussel populations in the Verdigris River of Oklahoma.

The detrimental effects of zebra mussels on native mussel communities are well documented (Burlakova *et al.* 2000, Martel *et al.* 2001, and Schloesser *et al.* 2006) although we found no difference in mussel abundances between sites upstream of Oologah Lake and sites downstream of the dam which are infested with the mussels. However, two relatively "good" sites greater than 20 km from the dam may have influenced these analyses. A number of native mussels were found to have byssal threads on their valves, indicating some degree of zebra mussel settling. It is possible that zebra mussel colonization of native mussels occurs during the spring, but the attached *Dreissena polymorpha* may be eliminated as water temperatures increase through the summer. The impoundment at Oologah Lake was designed for hypolimnetic releases, meaning if water releases occurred during summer, downstream habitats would receive cooler water. However, from August to November of 2006, there were no releases from the reservoir. Without this influence, it is reasonable that water temperature in these downstream reaches of the Verdigris River was similar to that in other streams in the area and approached 30 °C in mid summer. This temperature may be lethal to zebra mussels (Karatayev *et al.* 1998, Matthews and McMahon 1999, Elderkin and Klerks 2005) while unionids may be slightly more tolerant (Polhill and Dimock 1996). Additionally, monitoring of zebra mussel densities in Oologah Lake indicated a significant die-off beginning in late June 2006. Adult zebra mussel densities declined from nearly 150,000 /m^2 to <4,500 /m^2 from late June to September 2006 (Chad Boeckman, unpubl. data). Temperature-induced seasonal die-offs of zebra mussels in the Verdigris River may mean that zebra mussel fouling of native mussels does not reach high enough numbers to cause a significant effect. Other studies have reported mortality of unionids due to zebra mussel infestation within 2 to 8 years after initial zebra mussel colonization (Schloesser *et al.* 1996, 2006, Ricciardi *et al.* 1998, Schloesser and Masteller 1999).

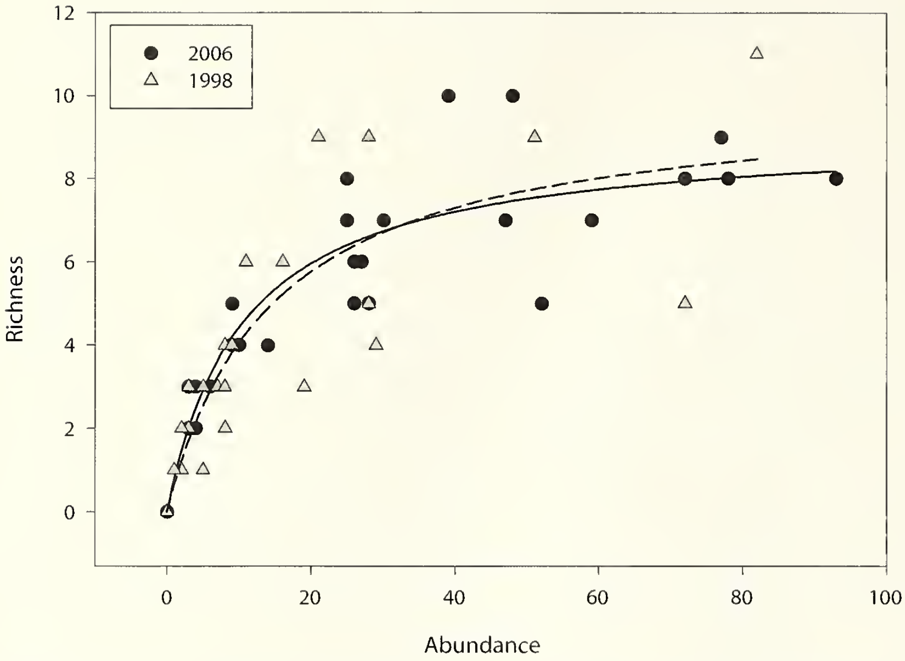

Figure 5. Richness (# of species/site) vs. abundance (total # of mussels found at that site) curves for the Vaughn (1998) survey (dashed line) and the current survey (solid line). For statistical analysis, these curves were linearized by log-transforming abundance. The resulting regression equations were $y = 4.332x + 0.1135$ for the present study and $y = 4.284x + 0.0257$ for Vaughn (1998).

Zebra mussels were first reported in Oologah Lake in June 2003 (Laney 2005), with numbers increasing steadily since that time. Negative effects on unionids due to zebra mussel colonization may still happen, if a cooler summer were to occur.

Several studies have shown that zebra mussels and native mussels can co-exist in habitats with fluctuating water depth, potential wave action, and substrate soft enough to allow native mussels to bury (Schloeser *et al.* 1997, Nichols and Amberg 1999, Bowers and De Szalay 2004, Strayer and Malcom 2007). The habitat below Oologah Lake does have some of these characteristics.

A potentially confounding factor in determining the effect of zebra mussels on unionid mussels is the influence of the Oologah Lake dam. The effects of impoundments on native mussel communities have been well established (Bogan 1993, Vaughn and Taylor 1999, Bednarek 2001, Sethi *et al.* 2004). For example, Vaughn and Taylor (1999) described a reduction in native mussel populations below an impoundment and found 20 km was needed for these populations to recover to pre-impoundment levels. In the current survey, mussel abundance downstream from the reservoir was positively correlated with distance from the dam. However, this relationship may have resulted from two sites (greater than 20 km from the dam) that harbored the greatest abundance of all 31 sites surveyed. Downstream sites less than 20 km from the dam generally had poor or less than average abundance, and two sites immediately downstream from the dam had no live mussels at all. Taxonomic richness for downstream sites was also positively correlated with distance from the dam although less distance from the dam was needed to "recover" as compared with abundance.

While it appears that zebra mussels are currently having little effect on native mussel richness and abundance below Oologah Lake, the consequences of long-term zebra mussel colonization on native mussels in this reach of the river are still unknown. For this reason, conservation efforts such as periodic defouling or propagation and reintroduction (Hallac and Marsden 2001, Strayer *et al.* 2004) to sites above Oologah Lake should be directed at species of special interest (and native mussels in general) that are currently located below Oologah Lake. *Quadrula cylindrica* should be a high priority for such efforts given its rather limited distribution in the Verdigris River (Obermeyer *et al.* 1997a, 1997b), the impoundment upstream and dredging activity downstream, and the relatively limited dispersal of its fish hosts.

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Spatial distribution of soft-bottom molluscs in the Ensenada de San Simón (NW Spain)

Eva Cacabelos[1], Patricia Quintas[2], and Jesús S. Troncoso[1]

[1] Área de Bioloxía Animal, Facultade de Ciencias do Mar, Universidade de Vigo, 36310 Lagoas-Marcosende, Vigo (Pontevedra), Spain, cacabelos@uvigo.es

[2] Biotecnología y Acuicultura, Instituto de Investigaciones Marinas de Vigo, Eduardo Cabello 6, 36208 Vigo, Spain

Abstract: Distribution and abundance of the molluscan fauna was studied in the intertidal and subtidal soft-bottoms of the Ensenada de San Simón (NW Spain). Depth, grain size, and total organic matter were the most important factors in determining distribution patterns of molluscs in this inlet. Three major malacological assemblages have been determined in the Ensenada de San Simón, two of them subdivided in two facies. In the intertidal area of the inlet, one facies (A1) was located in areas associated with seagrass meadows of *Zostera* spp. and was dominated by *Hydrobia ulvae* (Pennant, 1777) whereas the second facies (A2) had a high dominance of *H. ulvae*, *Cerastoderma edule* (Linnaeus, 1758), and *Tapes decussatus* (Linnaeus, 1758). An impoverished facies of this community was present in reduced, muddy bottoms (Group C). In the subtidal bottoms, one group (B1) was located in the central part of the inlet with *H. ulvae, Rissoa labiosa* (Montagu, 1803), *Turboella radiata* (Philippi, 1836), *Parvicardium exiguum* (Gmelin in Linnaeus, 1791), *Loripes lacteus* (Linnaeus, 1758), and *Abra nitida* (Müller, 1789) as characteristic species. A second facies (B2) was found in outer areas of the inlet, characterized by *Thyasira flexuosa* (Montagu, 1803), *Mysella bidentata* (Montagu, 1803), *Abra alba* (Wood, 1802), and *Nucula nitidosa* Winckworth, 1930.

Key words: macrofauna, *Macoma* community, *Abra alba* community, multivariate analysis, Atlantic Ocean

Several faunistic and ecological works on the macrobenthic communities of the Iberian and Galician coasts of Spain have been carried out in recent years (Anadón 1980, López-Jamar and Mejuto 1988, López-Jamar and Cal 1990, Troncoso and Urgorri 1991, Mazé *et al.* 1993, Junoy 1996). Benthic communities are considered good indicators of marine bottom conditions (Pearson and Rosenberg 1978, Grall and Glémarec 1997). The Ensenada de San Simón is located in the inner part of the Ría de Vigo, the southern-most of the Galician estuaries. These estuarine systems have been studied because of their great economic and social importance, including fisheries, raft mussel cultures, and shellfish resources.

Benthic communities of the Ría de Vigo have been analyzed since 1886 when Hidalgo published a list of marine species of the NW Spanish coast (Hidalgo 1917). Despite the abundance of studies in the Ensenada de San Simón (Nombela *et al.* 1995, Fernández Rodríguez *et al.* 1997, Álvarez-Iglesias *et al.* 2003), few researchers have analyzed patterns of benthic faunal spatial distribution, and none have quantified community structure. While the molluscan fauna was studied in other estuaries (Troncoso *et al.* 1996, 2005, Olabarría *et al.* 1998), the only previous studies in the Ría de Vigo were by Rolán (1983), Rolán *et al.* (1989), and Moreira *et al.* (2005).

Consequently, there is a need to improve our knowledge to ensure correct management and conservation in the area, especially due to Ensenada de San Simón being included in the Nature 2000 Network as a Special Conservation Zone. Therefore, the aim of the present study is to describe and quantify the malacofaunal communities and associations inhabiting intertidal and subtidal soft substrata throughout the Ensenada de San Simón. Characteristic and dominant species are studied to document their relationship with several environmental variables.

MATERIALS AND METHODS

Study area

The Ensenada de San Simón is located in the inner part of the Ría de Vigo (NW Spain), between 42°17′ and 42°21′N and between 8°37′ and 8°39′W (Fig. 1). The seagrasses *Zostera noltii* and *Zostera marina* cover the intertidal and shallow subtidal areas. Considerable freshwater input occurs in the inner-most part of the inlet which results in salinity fluctuations on a tidal and seasonal basis (Nombela and Vilas 1991). Culture of mussels on rafts is a common practice in large areas of the mouth of the inlet, and a small harbor is located in the mouth of the Alvedosa River.

Sampling and sediment laboratory analysis

Samples were collected during November and December 1999 from 29 sites (Fig. 1). Five samples were taken at each site, by means of a van Veen grab (0.056 m^2). Samples were sieved through 0.5 mm mesh and the retained material was fixed in 10% buffered formalin. Fauna was sorted from the sediment and preserved in 70% ethanol. Temperature

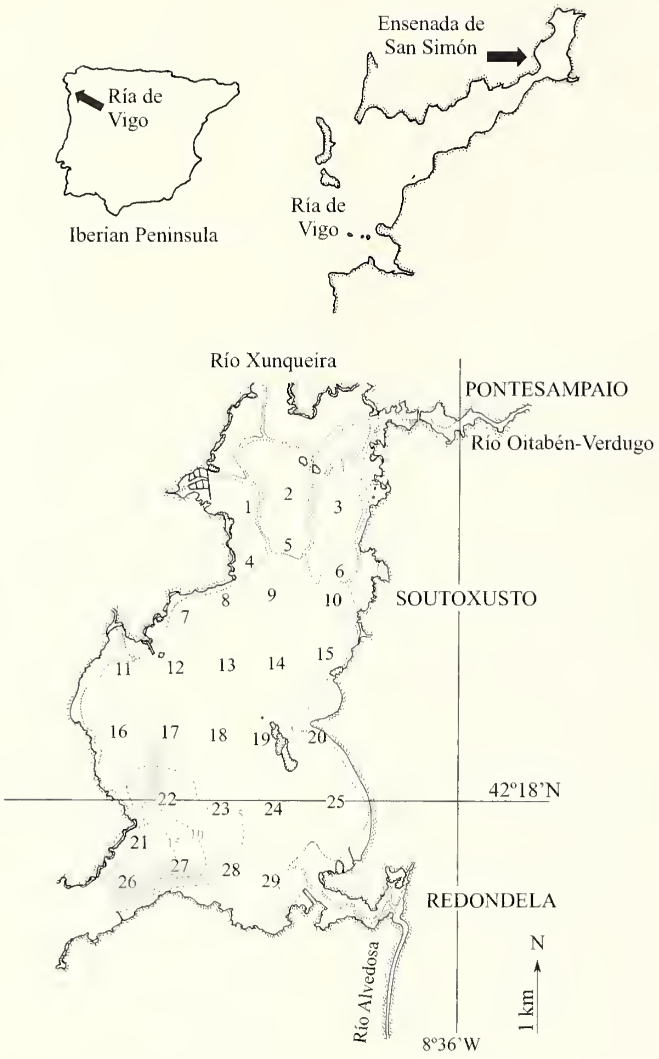

Figure 1. Study area showing the location of sampling sites in NW Spain. Gray figures represent mussel raft sites.

and pH were measured *in situ* from water and sediment samples taken from each site. An additional sediment sample was taken at each site for grain-size, calcium carbonate, and total organic matter content analyses. Granulometric fractions and sediment types were determined. The median grain size (Q_{50}, mm) and the sorting coefficient (S_o) were also calculated for each sample (Trask 1932). Calcium carbonate content (%) was estimated by sample treatment with hydrochloric acid, and total organic matter content (%) was estimated from the weight loss after combustion by placing samples in a furnace for 4 hours at 450 °C (Guitián and Carballas 1976, Parada *et al.* 1993).

Data analysis

Abundance (A), species richness (S), Shannon-Wiener's diversity index (H', $\log_2$) (Shannon and Weaver 1963), and Pielou's evenness index (J) (Pielou 1984) were determined for the five samples pooled in each site (0.28 m^2). Dominance was calculated as the percentage of the numbers of individuals belonging to one species with respect to the total number of specimens in that sample. Mollusc assemblages were based on the analysis of the species abundance data matrix by non-parametric multivariate techniques, using PRIMER software (Clarke and Warwick 1994). A similarity matrix was calculated, using the Bray-Curtis coefficient, after applying the fourth-root transformation to species abundance. From the similarity matrix, classification and ordination of the sites were analyzed: cluster analysis, algorithm UPGMA, and non-metrical multidimensional scaling, MDS. The SIMPER program was used to identify molluscan species that contributed to dissimilarity among groups.

Species were classified according to the constancy and fidelity indexes and to the fidelity-dominance product (Dajoz 1971). Relationships between abundance of molluscs and environmental variables were studied by means of the BIOENV procedure (PRIMER package) and canonical correspondence analysis (CANOCO package; ter Braak 1988). Environmental variables in percentages were transformed by logarithm (x+1) and all were normalized.

RESULTS

Sedimentary characterization

The soft bottoms of the Ensenada de San Simón were characterized by a predominance of muddy sediments with a high total organic matter and low calcium carbonate content (Appendix 1). Sandy sediments were present in tidal channels in the inner inlet where low total organic matter content was also found. Areas around the outer part of the inlet had muddy sands with a large gravel fraction composed of the mussel shells cultured there.

Molluscan fauna

A total of 24,605 individuals belonging to 68 species of molluscs (30 bivalves, 34 gastropods, 3 polyplacophorans, and 1 scaphopod) was sampled in the study area (Appendix 2). Gastropods were the most abundant group (88.92% of the total mollusc abundance) due mainly to large numbers of *Hydrobia ulvae* (Pennant, 1777). This hydrobiid showed densities of up to 34,946 individuals/m^2 in sandy bottoms of tidal channels and 14,800 individuals/m^2 in sediments colonized by *Zostera marina* and *Z. noltii* in the innermost part of the inlet. Other dominant gastropods were *Rissoa labiosa* (Montagu, 1803), *Turboella radiata* (Philippi, 1836), and *Chrysallida terebellum* (Philippi, 1844), mainly in intertidal and shallow bottoms. Bivalves were numerically the second most important group (10.99% of total) with the greatest numbers in the central area and at the mouth of the inlet.

The most abundant bivalves were *Cerastoderma edule* (Linnaeus, 1758) in sandy intertidal sediments and *Thyasira flexuosa* (Montagu, 1803), *Mysella bidentata* (Montagu, 1803), and *Abra alba* (Wood, 1802) in subtidal sediments. Polyplacophorans and scaphopods were found in small numbers (0.09%).

The highest abundances of molluscs were recorded at sites 6, 2, and 3 due to the high abundance of *Hydrobia ulvae*; the lowest were recorded at sites 24 and 28 (64.3-342.9 individuals/m^2). Sites 26, 27, and 22 at the mouth of the inlet showed the highest species richness (22-31). Only two species were found at sites 29 and 24, located in the mouth of the Alvedosa River. Shannon-Wiener's diversity index varied between H′ = 0.09 (site 3) and 3.75 (site 27), and evenness ranged from J = 0.04 (site 3) to 0.97 (site 24). Greatest H′ values were recorded in sites in the mouth of the inlet while the lowest values were found in intertidal sites with high numbers of *H. ulvae.*

Spearman's correlation coefficient indicated that depth was positively correlated with species richness ($r_s = 0.511$, $N = 29$, $P < 0.01$), diversity ($r_s = 0.639$, $N = 29$, $P < 0.01$), and evenness ($r_s = 0.568$, $N = 29$, $P < 0.01$), and negatively correlated with number of individuals ($r_s = -0.458$, $N = 29$, $P < 0.05$). Species richness was positively correlated with percent content in gravel ($r_s = 0.470$, $N = 29$, $P < 0.05$) and was negatively correlated with total organic matter ($r_s = -0.401$, $N = 29$, $P < 0.05$) and silt/clay content ($r_s = -0.376$, $N = 29$, $P < 0.05$). The number of individuals was positively correlated with percent calcium carbonate, median grain size ($r_s = 0.596$ and $r_s = 0.473$ respectively, $N = 29$, $P < 0.01$) and sand content ($r_s = 0.500$, $N = 29$, $P < 0.01$) and negatively correlated with silt/clay content ($r_s = -0.511$, $N = 29$, $P < 0.01$).

Multivariate analysis

The classification diagram based on abundance data showed three main groups: A, B, and C (Fig. 2). These three groups were further subdivided into five subgroups. Group A1 had muddy sediment, sandy mud, and very coarse sand. Group A2 was composed of coarse sand and muddy sand bottoms. Group B1 was comprised of muddy sediments (mud and sandy mud to muddy sand). Group B2 sites had mud and muddy sand bottoms. Group C sites had muddy sediments. This classification agreed with ordination of sites obtained through non-metrical multidimensional analysis (Fig. 3).

Water depth presented the highest correlation with faunistic data according to the BIOENV procedure (Spearman's rank correlation $\rho_w = 0.312$). It was followed by the combination of depth and median grain size ($\rho_w = 0.201$), and that of bottom water temperature, very fine sand, fine silt, clay, and depth ($\rho_w = 0.192$).

In the canonical correspondence analysis, the first two axes accounted for 44.4% of the total variance of species-environment relationships and 34.0% of the species variance (Table 1). Species-environment correlations close to 1 indicated that abiotic variables were correctly chosen (ter Braak 1988) while the maximum eigenvalue reached in the first axis was close to the optimal 0.7. Bottom water and sediment temperature, coarse and fine silts, clay, and calcium carbonate showed the highest correlations with axis I; however, correlations with other axes were less significant. Forward selection indicated water depth as the variable explaining most of the variance in the species data ($F = 3.82$, $P < 0.01$) as well as bottom-water temperature ($F = 1.82$, $P < 0.05$), total organic matter ($F = 1.73$, $P < 0.05$), clay ($F = 1.63$, $P < 0.05$), and very fine sand ($F = 1.91$, $P < 0.05$). The scatter diagram showed an ordination of sites following a gradient of depth and grain size (Fig. 4). Sites from Group A1 and A2 were progressively distributed along the negative part of axis I, in intertidal or shallow waters with coarse sediments; sites from group B1 and B2 were deeper with greater content in finer sedimentary fractions.

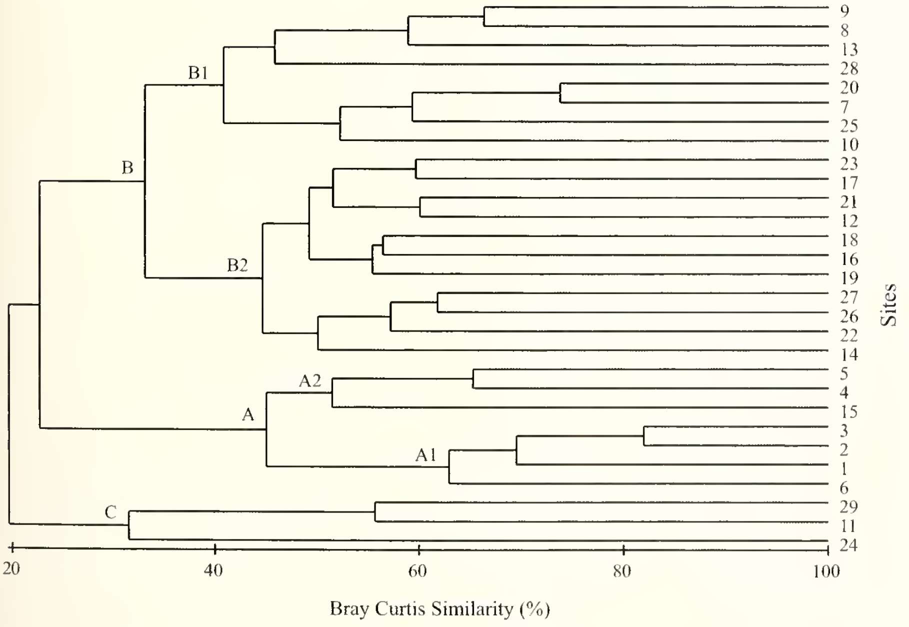

Figure 2. Dendrogram showing the groups and subgroups considered.

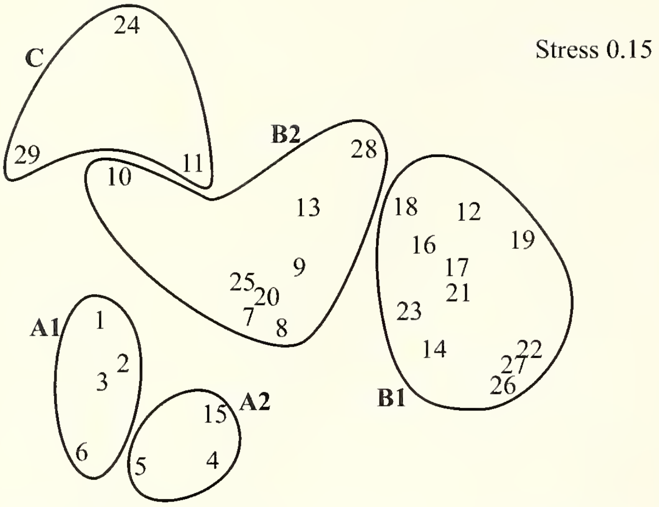

Figure 3. Ordination diagram representing the assemblages considered in the classification analysis. Numbers refer to sites. Stress indicates the goodness-of-fit for the model: the smaller the stress, the better the representation.

The analyses suggested that the distribution of molluscan fauna is mainly determined by depth, organic matter content, and grain size gradients.

Description of assemblages

Classification and ordination analyses differentiated five different assemblages (Tables 2-3, Fig. 5). Group A located in the inner-most part of the inlet, in intertidal sediments close to the mouth of the Oitabén-Verdugo and Xunqueira Rivers. The most abundant species was *Hydrobia ulvae*, mainly in bottoms colonized by *Zostera noltii.* Subgroup A1 was comprised of four intertidal sites located along the northern border of the inlet in heterogeneous sediments. These bottoms exhibited low species richness (S = 11). The seagrasses *Zostera marina* and *Z. noltii* were spread across most of these bottoms. The most characteristic species according to values of fidelity-dominance product (F × D) were *H. ulvae*, *Chrysallida terebellum*, and *Cerastoderma edule.* This area had the smallest mean diversity value due to the high dominance of *H. ulvae.* SIMPER analysis showed that *H. ulvae* and *C. edule* were the species with a greater similarity contribution (75%) for this group. Subgroup A2 was located in the intertidal sandy bottoms with the greatest content in coarse sand (23.2% ± 17.5, mean ± *SD*) and the greatest median. A total of 28 species was found, and the most characteristic, according to F × D, were *H. ulvae*, *C. edule*, *Tapes decussatus* (Linnaeus, 1758), *Ostrea edulis* Linnaeus, 1758, *C. terebellum*, and *Retusa truncatula* (Bruguière, 1792). In accordance with the SIMPER analysis, Group A2 was mainly defined by *H. ulvae*, *C. edule*, *R. truncatula*, *T. decussatus*, and *Lepton nitidum* Turton, 1822.

Group B was present in muddy bottoms, from intertidal areas to 28 m depth in the mouth of the inlet. Species richness increased from sites in inner areas towards the mouth. Sediments were mainly composed of silt and clay (>50%). Subgroup B1 was defined in shallow sediments in the center of the inlet (0-4.7 m depth). Sites 10 and 20 had *Zostera marina* meadows. This subgroup had 27 species and showed the greatest mean value for evenness. The F × D and dominance values indicated that the most characteristic species were *Hydrobia ulvae*, *Rissoa labiosa*, *Turboella radiata*, *Parvicardium exiguum* (Gmelin in Linnaeus, 1791), *Loripes lacteus* (Linnaeus, 1758), *Abra nitida* (Müller, 1789), and *Chrysallida terebellum.* According to the SIMPER analysis, Group B1 was characterized by *H. ulvae*, *C. terebellum*, *T. radiata*, and the bivalve *P. exiguum.* Subgroup B2 was located in the external part of the inlet, in subtidal bottoms (3.7-28.2 m). These bottoms showed the highest mean value for calcium carbonate. Fifty-nine molluscan species were found and many of them showed great Fidelity values. Among the species with highest values of F × D were *Thyasira flexuosa*, *Mysella bidentata*, *Calyptraea chinensis* (Linnaeus, 1758), *Abra alba*, *Nucula nitidosa* Winckworth, 1930, and *Hyala vitrea* (Montagu, 1803). Other species with high values of constancy and fidelity were *Myrtea spinifera* (Montagu, 1803), *Chrysallida fenestrata* (Jeffreys, 1848), and *Corbula gibba* (Olivi, 1792). The bivalves *T. flexuosa*, *M. bidentata*, *A. alba*, *N. nitidosa*, and gastropod *C. chinensis* defined group B2, according to SIMPER.

Group C was mainly characterized by *Hydrobia ulvae* (86% of cumulative similarity). This group was composed of muddy sites close to the mouth of several small rivers and Redondela harbor. Sediments were predominantly composed of silt and clay with low carbonate content, and the greatest values of total organic matter in the inlet. Only 8 species were found and densities were less than in other groups (1202.50 ± 898.21 individuals/m^2). The species with highest values of F × D were *H. ulvae*, *Turboella radiata*, and *Loripes lacteus*, while the group was mainly characterized by *H. ulvae* (86% of cumulative similarity).

DISCUSSION

The distribution of the molluscan fauna seemed to be mainly determined by depth, organic matter content, and grain-size in the Ensenada de San Simón, NW Spain. Intertidal and shallow sediments in inner channels were mostly sandy and then became increasingly muddy towards the deeper bottoms in the center and at mouth of the inlet. The lack of strong currents in the greater part of the inlet and the very common culture of mussels on rafts were responsible

Table 1. Canonical correspondence analysis for the Ensenada de San Simón.

Axes	I	II	III	IV	Total inertia
Eigenvalues	0.680	0.361	0.200	0.170	3.061
Species-environment correlations	0.984	0.914	0.976	0.935	
Cumulative percentage variance of species data	22.2	34.0	40.5	46.1	
Sum of all unconstrained eigenvalues					3.061
Sum of all canonical eigenvalues					2.347

for the progressive increase of fine particles and organic matter content in the sediment. The silt/clay contents found in intertidal areas were higher than described by Nombela and Vilas (1986-1987). This situation is not surprising. On one hand, the presence of *Zostera* spp. stabilizes the sediment (Nombela *et al.* 1995); on the other hand, intense culture of mussels on rafts is located at the study sites (Fig. 1) and in the greater part of Galician estuaries. This culture is an important human disturbance since it produces large quantities of fecal pellets that substantially modify sediment composition, increasing the clay fraction (Nombela *et al.* 1987, León *et al.* 2004). The granulometric change has an important impact on the benthos community and also affects trophic structure (Abella *et al.* 1996, Conde and Domínguez 2004). Moreover, anoxic situations can be produced by pellet sedimentation with high organic matter content under the rafts (Tsuchiya 2002). Since the biodeposits produced by one raft could reach 190 kg dry weight d^{-1} (Cabanas *et al.* 1979) and in San Simón there are 76 rafts, the effect of this activity reduces the depth in the inlet between 0.5 and 2 cm y^{-1}. However, this culture could be considered also as an important depurator because mussels ingest high quantities of particulate organic matter (Fernández Rodríguez *et al.* 1997).

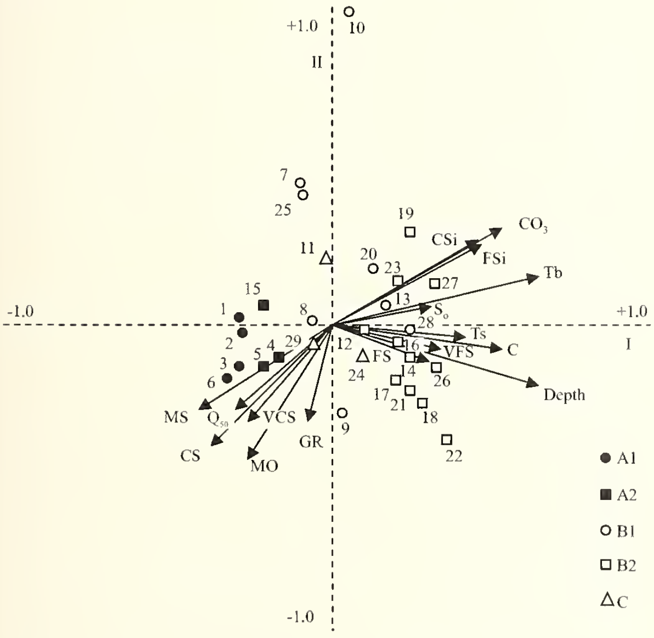

Figure 4. Canonical correspondence analysis ordination of environmental variables and sites in relation to axes I and II, representing the groups and subgroups in the classification analysis. Tb, temperature of bottom-water; Ts, temperature of surface-water; CO_3, carbonates; OM, organic matter; Q_{50}, median grain size; S_o, sort coefficient; GR, gravel; VCS, very coarse sand; CS, coarse sand; MS, medium sand; FS, fine sand; VFS, very fine sand; CSi, coarse silt, FSi, fine silt; C, clay.

Species richness, diversity, and evenness were greater on subtidal bottoms than on intertidal areas. This is a consequence of the stressful conditions that aquatic fauna must tolerate in intertidal areas: this fauna is subjected to important environmental changes such as extreme temperatures, desiccation, or rough conditions on the floor (Kikuchi 1987). The fauna in these intertidal sediments in the Ensenada de San Simón must also tolerate changes in salinity due to the freshwater input from several rivers (Vilas *et al.* 1995). Salinity fluctuations may greatly influence the species richness and the species composition of the community (Planas and Mora 1987), benefiting euryhaline species such as *Hydrobia ulvae*. As well as in Ensenada de San Simón, large densities of *H. ulvae* are common in inner areas of other Galician estuaries having organic pollution (Planas *et al.* 1984, Currás and Mora 1990, Junoy 1996, Olabarría *et al.* 1998). This species has a broad range of food sources since it is a detritivore on organic remains and fecal pellets (Jacobs *et al.* 1983) or grazes on microalgae (Muus 1967) which may explain the large numbers in these sediments.

Intertidal sediments colonized by *Zostera noltii* and *Zostera marina* showed low diversities of molluscs. Seagrass meadows provide a complex habitat that may be colonized by many species (Somersfield *et al.* 2002), stabilizing the sediment and providing protection to potential prey. However, low diversities characterize these meadows in San Simon since they are located in areas subjected to abrupt salinity changes, a major limiting factor for non-euryhaline species (Planas and Mora 1987, Junoy 1996). Subtidal sediments show more stable conditions in terms of salinity and currents (Nombela *et al.* 1987). However, the sites with the lowest species richness and abundance were the muddy bottoms close to the mouth of freshwater channels and in the harbor. Fine and homogeneous sediments have been related

Table 2. Summary of biotic and physical characteristics of the associations. Values: mean ± *SD*. Depth <2 m is intertidal; Q_{50}, median grain size; Bt, bottom type (VCS, Very coarse sand; CS, Coarse sand; MS, Muddy sand; M, Mud); OM, percent total organic matter content; CO_3, percent carbonate content. Faunistic parameters at each site per m^2: S, species richness; A, abundance; J, Pielou's evenness; H′, Shannon-Wiener's diversity index.

Group	A1	A2	B1	B2	C
Depth (m)	1.60 ± 0.00	1.73 ± 0.11	3.10 ± 0.88	9.04 ± 7.77	3.23 ± 1.10
Q_{50}	0.31 ± 0.56	0.77 ± 0.38	0.06 ± 0.08	0.15 ± 0.45	0.01 ± 0.00
% Gravel	5.85 ± 10.23	17.08 ± 10.82	3.23 ± 4.12	6.20 ± 11.66	0.03 ± 0.05
% Sand	45.83 ± 26.37	77.58 ± 12.52	36.46 ± 26.40	26.32 ± 10.00	17.72 ± 12.26
% Silt/Clay	48.31 ± 34.88	5.34 ± 2.50	60.30 ± 28.67	67.49 ± 20.35	82.25 ± 12.31
Bt	M-VCS	MS-CS	M-MS	M-MS	M
% OM	17.45 ± 11.42	2.69 ± 1.63	17.59 ± 11.61	17.17 ± 4.95	20.75 ± 6.11
% CO_3	7.30 ± 3.13	7.23 ± 0.96	4.74 ± 1.21	8.04 ± 10.89	4.44 ± 0.37
S	6.75 ± 1.5	15.67 ± 5.03	11.62 ± 3.25	17.73 ± 6.35	3.67 ± 2.89
A	159589.29 ± 139930.00	26916.79 ± 17955.36	8218.57 ± 7489.64	8207.14 ± 6280.71	1202.50 ± 898.21
J	0.06 ± 0.22	0.41 ± 0.17	0.69 ± 0.22	0.62 ± 0.14	0.61 ± 0.33
H′	0.16 ± 0.06	1.56 ± 0.63	2.43 ± 0.82	2.56 ± 0.82	0.93 ± 0.59

Table 3. Characteristic species of each group according to SIMPER and F × D values are listed indicating their constancy (Ct, constant; C, common; VC, Very common) and fidelity (Ex, Exclusive; El, Elective; Pr, Preferential; Ac, Accessory; Oc, Occasional).

A1	A2	B1	B2	C
Hydrobia ulvae (Ct, Oc)	*H. ulvae* (Ct, Oc)	*H. ulvae* (Ct, Oc)	*Calyptraea chinensis* (Ct, Ac)	*H. ulvae* (Ct, Oc)
Cerastoderma edule (Ct, Oc)	*Retusa truncatula* (Ct, Pr)	*Turboella radiata* (VC, Ac)	*Nucula nitidosa* (Ct, El)	*T. radiata* (VC, Ac)
Chrysallida terebellum (Ct, Oc)	*C. edule* (Ct, Oc)	*C. terebellum* (Ct, Oc)	*Thyasira flexuosa* (Ct, Ac)	*L. lacteus* (C, Oc)
	Tapes decussatus (Ct, El)	*Parvicardium exiguum* (VC, Ac)	*Mysella bidentata* (Ct, Ac)	
	Lepton nitidum (Ct, El)	*Rissoa labiosa* (C, Oc)	*Abra alba* (Ct, Ac)	
	Ostrea edulis (C, El)	*Loripes lacteus* (VC, Oc)	*Hyala vitrea* (C, Ex)	
	C. terebellum (VC, Oc)	*Abra nitida* (C, Pr)	*Myrtea spinifera* (C, Ex)	
			Chrysallida fenestrata (C, Ex)	
			Corbula gibba (C, Ex)	

to low diversities: as the grain size decreases, there are restrictions in interstitial space and oxygen diffusion (Olabarría *et al.* 1998). In general, diversity values observed in the Ensenada de San Simón were high (1.92 ± 1.13) in comparison to other Galician estuaries with a predominance of muddy bottoms (López-Jamar and Mejuto 1985). Mean diversity values were generally greater in exposed estuaries with sandy and more heterogeneous sediments, as Ría de Ares-Betanzos (2.38 ± 0.82, mean ± *SD*, Troncoso and Urgorri 1991), Ensenada de Baiona (2.37 ± 0.74, mean ± *SD*, Moreira *et al.* 2005), and Ría de Aldán (2.77 ± 0.84, mean ± *SD*, Lourido *et al.* 2006).

The assemblages in the Ensenada de San Simón determined by the different multivariate approaches could be described as classic communities or facies. The assemblage in the intertidal areas corresponding to Group A had the typical fauna of the small *Macoma* community (community of *Cerastoderma edule-Scrobicularia plana*). The facies located in areas associated with meadows of *Zostera* spp. (A1) was dominated by *Hydrobia ulvae*; the second facies (A2) presented a high dominance of *H. ulvae*, *C. edule*, and *Tapes decussatus*. Similar faunal assemblages have been reported from other intertidal and shallow bottoms in the Galician estuaries (Cadée 1968, Anadón 1980, Mora 1982, Troncoso and Urgorri 1991, Mazé *et al.* 1993). In estuarine bottoms with high organic content cited by Junoy (1996) and Olabarría *et al.* (1998), the assemblage tends to be dominated by *H. ulvae*. An impoverished facies of a small *Macoma* community was present in reduced muddy bottoms (Group C). In this case, salinity fluctuations coupled with effects from

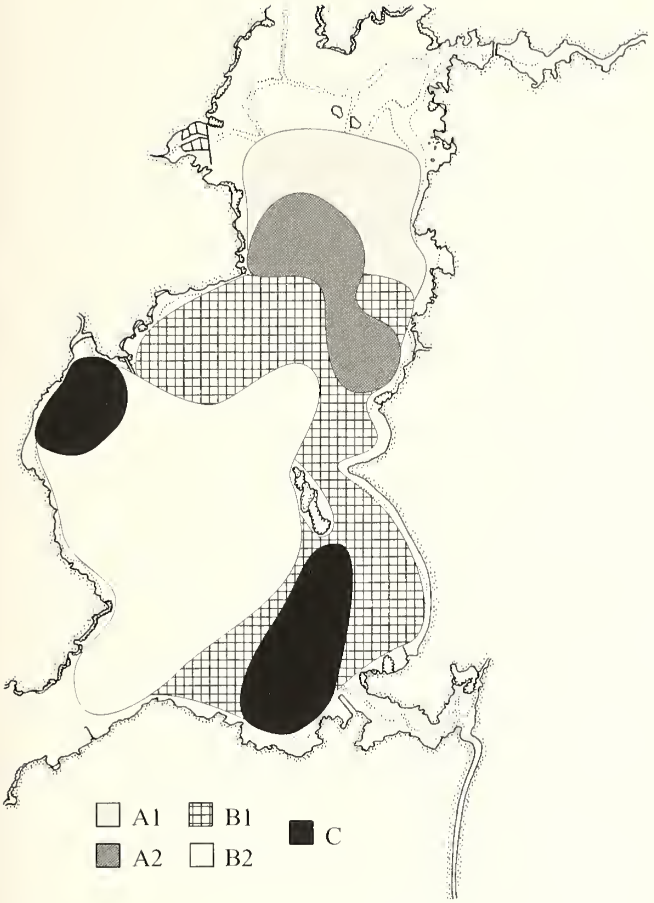

Figure 5. Spatial distribution of molluscan assemblages in the Ensenada de San Simón, Spain.

human activities, such as organic enrichment and sewage disposal, may be responsible for the scarce malacological fauna near shore.

Group B can be ascribed to the *Abra alba* community (Petersen 1918) from muddy bottoms (Lastra *et al.* 1990). The facies present in subgroup B1 had a transitional fauna that was between that of the small *Macoma* community (*e.g.*, *Hydrobia ulvae*, *Rissoa labiosa*), and of a typical *A. alba* community, with species that tend to be more abundant in muddier sediments, such as the bivalves *Abra nitida*, *Mysella bidentata*, and *Thyasira flexuosa*. The facies in deeper bottoms of Subgroup B2 was characterized by the greater dominance of *T. flexuosa* and *M. bidentata*. Similar assemblages, showing transitional faunas between typical "communities" (as in Thorson 1957) both in composition of species and numbers for any given species according to gradients in depth and granulometry, have been reported by Sánchez-Mata and Mora (1999), Moreira *et al.* (2005), and Lourido *et al.* (2006) in a variety of muddy bottoms of Galicia. *T. flexuosa* has been considered as an opportunist in disturbed situations (López-Jamar and Mejuto 1988) and prefers muddier sediments (Moreira *et al.* 2005), as was the case in San Simón. López-Jamar and Parra (1997) detected high faunal abundances in similar bottoms of the Galician coasts.

In conclusion, the most important factors in determining distribution patterns of molluscs in the Ensenada de San Simón were depth, grain size, and total organic matter content. The presence of muddy sediments in this inlet is a consequence both of the hydrodynamic regime, which deposits finer fractions in subtidal areas, and mussel bivalve culture. Similarities both in sediment and faunistic composition have been reported by Mora *et al.* (1989) in Ensenada de Lourizán, López-Jamar and Mejuto (1988) in A Coruña harbor, Lourido *et al.* (2006) and Sánchez-Mata and Mora (1999) in inner areas of Ría de Aldán and Ares-Betanzos, or Olabarria *et al.* (1998), Mora (1982), and Junoy (1996) in the *Zostera* meadows in Ensenada de O Baño, O Grove, or Ría de Foz, respectively.

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Appendix 1. Depth (<2 m is intertidal) and characteristics of sediments at each site. Q_{50}, median grain size; CO_3, percent carbonate content; OM, percent total organic matter content (% based on dry mass).

Site	Depth (m)	Q_{50}	Gravel (%)	Sand (%)	Silt/Clay (%)	Bottom type	OM (%)	CO_3 (%)
1	1.6	0.01	0.1	16.8	83.1	Mud	26.52	5.52
2	1.6	0.01	2.2	32.9	64.9	Mud	23.30	5.60
3	1.6	0.08	0.0	56.7	43.3	Sandy mud	19.05	6.12
4	1.6	0.32	17.8	74.0	8.2	Muddy sand	2.16	6.00
5	1.8	1.25	30.0	64.3	5.7	Muddy sand	4.90	7.33
6	1.6	1.15	21.1	76.8	2.1	Very coarse sand	0.95	11.98
7	3.4	0.15	0.3	74.3	25.4	Sandy mud	3.95	6.31
8	3.2	0.04	0.6	35.9	63.5	Mud	10.88	5.80
9	2.9	0.01	0.9	27.7	71.4	Mud	18.12	4.28
10	2.9	0.01	0.0	2.3	97.7	Mud	36.93	4.28
11	3.6	0.01	0.0	8.9	91.1	Mud	26.50	4.81
12	3.8	0.01	1.1	19.2	79.7	Mud	19.93	2.12
13	3.5	0.01	3.0	23.0	74.0	Mud	23.00	2.36
14	4.6	0.01	7.1	24.4	68.5	Mud	19.78	2.28
15	1.8	0.74	3.5	94.4	2.1	Coarse sand	1.00	8.35
16	4.2	0.01	1.0	15.5	83.5	Mud	21.47	4.53
17	3.7	0.02	4.7	31.1	64.2	Mud	18.93	5.90
18	4.5	0.01	1.9	20.0	78.1	Mud	15.20	4.52
19	4.7	0.01	0.0	13.9	86.1	Mud	21.05	4.53
20	2.6	0.21	11.8	77.7	10.5	Muddy sand	1.80	4.85
21	18	0.01	0.6	26.3	73.1	Mud	19.50	4.61
22	10.4	0.01	1.0	37.4	61.6	Mud	12.98	5.51
23	5.9	0.01	1.2	25.2	73.6	Mud	22.17	5.40
24	4.1	0.01	0.0	12.6	87.4	Mud	21.42	4.07
25	1.6	0.01	6.8	31.8	61.4	Mud	23.72	5.47
26	28.2	1.50	40.2	48.3	11.5	Muddy sand	7.22	40.46
27	11.5	0.01	9.3	28.2	62.5	Mud	10.60	8.61
28	4.7	0.01	2.4	19.0	78.6	Mud	22.32	4.61
29	2	0.01	0.1	31.7	68.2	Mud	14.33	4.45

Appendix 2. Faunistic parameters at each site: S, species richness; A, total abundance (individuals/m^2); J, Pielou's evenness; and H′, Shannon-Wiener's diversity index.

Site	S	A	J	H′
1	6	2,982.1	0.09	0.24
2	8	15,014.3	0.05	0.14
3	5	10,246.4	0.04	0.09
4	15	821.4	0.61	2.37
5	11	2,139.3	0.42	1.47
6	8	35,592.9	0.05	0.16
7	13	1,385.7	0.48	1.77
8	17	1,189.3	0.62	2.54
9	13	207.1	0.88	3.26
10	6	2,178.6	0.29	0.75
11	7	157.1	0.54	1.51
12	9	157.1	0.60	1.91
13	11	107.1	0.90	3.10
14	21	2,467.9	0.63	2.78
15	21	5,114.3	0.19	0.83
16	13	653.6	0.34	1.25
17	16	925.0	0.47	1.90
18	13	303.6	0.57	2.10
19	12	717.9	0.49	1.75
20	11	396.4	0.83	2.88
21	16	332.1	0.73	2.91
22	31	1,117.9	0.74	3.65
23	18	796.4	0.68	2.84
24	2	17.9	0.97	0.97
25	13	1,014.3	0.64	2.37
26	22	1,046.4	0.74	3.28
27	24	510.7	0.82	3.75
28	9	96.4	0.88	2.79
29	2	185.7	0.32	0.32

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Introduction to the symposium "Advances in Chiton Research"*

Douglas J. Eernisse

Department of Biological Science, California State University, Fullerton, California 92834, U.S.A., deernisse@fullerton.edu

The present volume features contributions from participants of the symposium, "Advances in Chiton Research," in Seattle, Washington on 31 July 2006. As the organizer for this symposium, I was impressed with the willingness of national and international authorities or students whose diverse research involves chitons to participate in these meetings. The symposium was a tremendous success and compared favorably to four previous meetings of international scope that were devoted to chitons: (1) 1987 AMS symposium on "Biology of the Polyplacophora" in Key West, Florida (see *American Malacological Bulletin* 6(1), 1988); (2) 1st International Chiton Symposium, 1991, Adelaide, Australia (see *Journal of the Malacological Society of Australia* 13, 1992); (3) the 4th International Workshop on Malacology devoted to Polyplacophora, 2001, Menfi, Sicily, Italy (see *Bollettino Malacologico* Supplemento 5: I-IV, [2003] 2004); and (4) 2nd International Chiton Symposium, 2003, Tsukuba, Japan (see *Venus* 65(1-2), 2006). The participants of the present symposium (Fig. 1) featured 14 speakers, of whom half were international, and 10 posters devoted to chitons. Including all co-authors, there were 39 total contributors to the symposium and about a third of these were students.

Research on chitons is central to many aspects not only of malacology but also of zoology, paleontology, evolutionary biology, molecular systematics, molecular evolution, physiology, and ecology (reviewed by Schwabe and Wanninger 2006, Eernisse 2007, Todt *et al.* 2008). The present collection of articles reflects this integrative role for contemporary chiton research. Some of the symposium speakers are not represented here because they have already published articles related to their talks in other journals, including Jean-Bernard Caron (Caron *et al.* 2006a, 2006b), Ryan Kelly (Kelly and Eernisse 2007, 2008, Kelly *et al.* 2007), and Enrico Schwabe (Schwabe 2008). Lesley Brooker ("Genes and biomineralization in the radular teeth of chitons") and Bruce Runnegar ("Paleontological evidence for the origin of valves in polyplacophoran molluscs") gave insightful presentations and have contributed as co-authors on articles in this volume. Bernie Lieb and his coauthors have continued to elucidate the molecular evolution and systematics of molluscan hemocyanin (*e.g.*, Bergmann *et al.* 2007), and his forthcoming collaborative studies on chiton hemocyanin as a promising new phylogenetic marker are eagerly anticipated. Those who have contributed articles for the present volume still represent an impressive cross-section of the diverse, ongoing research on chitons.

Pojeta and DuFoe (this volume) have extended what is known about the earlier described Ordovician spiny chiton, *Echinochiton dufoei* Pojeta, Eernisse, Hoare, and Henderson, 2003. This fossil has already figured prominently in the ongoing debate on the disparity of Paleozoic chitons, including whether the geologically younger multiplacophorans diverged from within chitons or from an earlier "stem chiton" ancestor, and whether certain Cambrian "problematica" with disputed affinities, such as *Wiwaxia* Walcott, 1911, halkierids, and *Odontogriphus* Conway Morris, 1976 could potentially be close relatives of chitons. The four previously known *E. dufoei* specimens were already remarkable for their articulated preservation but details of the anterior portion of the animal were still unknown. After additional monumental collecting effort by co-author Jimmie DuFoe, resulting in the discovery of even better fossil examples that were also displayed in a special session at the symposium, Pojeta and DuFoe are now able to provide details of the anterior portion. They show that the anterior portion has the same striking hollow girdle spines found surrounding the rest of the animal. The authors also reconsider the significance of *E. dufoei* in discussions of molluscan and polyplacophoran evolution.

Shaw *et al.* (this volume) have contributed an extremely useful description of methods they used to analyze radular tooth formation and biomineralization, ensuring minimum deformation of the fragile associated tissue layers involved in biomineralization processes. Based on Jeremy Shaw's Ph.D. research, the authors have employed multiple state-of-the-art electron microscopy approaches to analyzing biomineralization processes in chitons, the results of which are being published elsewhere (*e.g.*, Shaw *et al.*, 2008). The exquisite results achieved by these authors reflect not only the con-

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* From the symposium "Advances in Chiton Research" presented at the joint meeting of the American Malacological Society and Western Society of Malacologists, held 29 July to 3 August 2006 in Seattle, Washington.

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Figure 1. Attending participants in the “Advances in Chiton Research” symposium, 31 July 2006 in Seattle, Washington. Numbers correspond to the inset key: 1. Stephaney Puchalski; 2. Albert Rodriguez; 3. Jeremy Shaw; 4. John Pojeta, Jr.; 5. Roger Clark; 6. Ryan Kelly; 7. Alejandro Herrera-Moreno; 8. Liliana Betancourt; 9. Donald Cadien; 10. Hiroshi Saito; 11. Lesley Brooker; 12. Doug Eernisse; 13. Mike Vendrasco; 14. John Buckland-Nicks; 15. Jimmie DuFoe; 16. Julia Sigwart; 17. Anel Ramirez Torres; 18. Klaus Streit; 19. Bernie Lieb; 20. Christine Fernandez; 21. Bruce Runnegar; and 22. Jean-Bernard Caron.

siderable contributions by Shaw but also the high quality of the electron microscope facility at Murdoch University, Perth, Western Australia, headed by co-author David Macey, and notably drawing on the considerable expertise of Shaw’s mentor, co-author Lesley Brooker. Besides a detailed examination of potential fixation artifacts, with implications for interpreting electron micrographs, I was especially impressed by the simple method for cleaning a radula using a high-pressure jet of water. The clever adaptation of a disposable pipet tip not only allows for avoiding artifacts associated with applying alkaline treatment but also results in the most pristine images of a chiton radula that I have ever seen.

Sigwart (this volume) has extended what has long been recognized as a phylogenetically informative set of traits, the position of the gill rows relative to the foot, the nephridiopores, and the gonopores, and also characteristics and the number of the gills within each gill row, to reveal unexpected variation in the most poorly known of all chiton taxa: the mostly deep-water Lepidopleurida (*sensu* Sirenko, 2006; alternatively as Lepidopleurina). Her present contribution and her ongoing molecular and morphological investigations are welcome additions to the scant literature on lepidopleurid chitons.

Vendrasco *et al.* (this volume) have investigated the phylogenetic utility of the aesthete (or esthete) canal morphology in Mopaliidae, testing between different expectations implied by either its conventional classification or the conflicting arrangement predicted by molecular results (Kelly and Eernisse 2008, Eernisse, unpubl. data). This is a significant change because it implies that Mopaliidae, as recently reformulated (*e.g.*, Eernisse *et al.* 2007), had a relatively recent origin and a dramatic subsequent diversification while largely confined to the northern Pacific Ocean. Based on the pattern of innervation of esthetes, Vendrasco *et al.* provide independent corroboration generally agreeing with the molecular arrangement. Moreover, they have further demonstrated the phylogenetic utility of considering esthete innervation patterns across chitons.

Clark (this volume) has contributed two significant taxonomic articles here, the first clarifying the taxonomic status a north/south species pair of common, but confusing, shallow-water chitons found along western North America. In agreement with recent morphological and molecular treatments (Eernisse *et al.* 2007, Kelly and Eernisse 2008), he has formally revived *Mopalia kennerleyi* Carpenter, 1864 from obscurity for the northern species (Alaska to northern California) and has restricted *Mopalia ciliata* (Sowerby, 1840) to the south, occurring no further north than northern California. Clark's second contribution introduces two new species discovered by recent exploration of the deep-water habitat of the Monterey Sea Canyon and also restores full generic status for members of *Tripoplax* Berry, 1919, to which the new species are assigned.

Puchalski *et al.* (this volume) have assembled a comprehensive database of nominal fossil chiton species, made available on-line (http://biology.fullerton.edu/deernisse/fossilchitons/) in association with this publication. They have used this database to investigate potential sampling biases that have likely affected perceptions of the chiton fossil record.

Buckland-Nicks (this volume) provides an overview and new analysis, reviewing phylogenetic inferences that have been drawn from comparing chiton eggs, egg hull coverings, sperm morphology, and egg-sperm interactions during fertilization. As he and his colleagues have continued to demonstrate in publications featuring splendid electron microscopy (*e.g.*, Buckland-Nicks and Brothers 2008), attention to chiton gametes and their interaction is highly informative for chiton phylogenetics.

Saito *et al.* (this volume) have provided a thorough description of three newly discovered chiton species found near hydrothermal vents and cold seeps around Japan. The authors also consider whether these and other chitons reported from similar habitats are necessarily associated with these chemosynthetic environments.

Finally, I have contributed (Eernisse, unpubl. data) a preliminary phylogenetic analysis of worldwide chitons based on about 350 partial sequences of the mitochondrial 16S ribosomal DNA gene. While this is planned to be the first phase before an eventual multi-locus analysis including these same taxa, the 16S gene appears to be relatively effective in both separating chiton species and in providing a higher-level inference of relationships that agrees well with recent cladistic morphological analyses. The taxon sampling in this study is much more extensive than in the only previous DNA-based analysis of chiton phylogeny (Okusu *et al.* 2003). This has allowed a more complete inference of relationships across chitons, with important phylogenetic implications that mostly agree with, but also challenge, certain aspects of our best available classifications of living chitons (*e.g.*, Sirenko 2006).

I thank the 2006 AMS/WSM President, Roland Anderson (Seattle Aquarium), for enlisting me as organizer for this symposium. I am grateful to AMS and WSM for helping with registration costs for the day of the symposium and travel-cost assistance.

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New information about *Echinochiton dufoei*, the Ordovician spiny chiton*

John Pojeta, Jr.[1] and Jimmie DuFoe[2]

[1] U.S. Geological Survey and Department of Paleobiology, Museum of Natural History, Smithsonian Institution, Washington, D.C. 20560, U.S.A., pojetaj@si.edu

[2] 417 Grove Street, Rockton, Illinois 61072, U.S.A., jdufoe2001@yahoo.com

Abstract: *Echinochiton dufoei* Pojeta *et al.*, 2003 is now known from seven specimens. The new material shows the anterior end and allows for a full reconstruction of the animal. The hollow spines are circumsomal; they were flexible and perhaps moveable in rotary anterior-posterior directions. Possible functions for the hollow spines are discussed. The relationships of *E. dufoei* to other chitons and to other molluscs and mollusc-like organisms are presented.

Key words: Mollusca, Polyplacophora, Fossil, Wisconsin

Echinochiton dufoei Pojeta *et al.*, 2003 was based on four partial specimens, three of which are parts and counterparts. None of these preserved the anterior hollow spines or fully preserved the anterior valves, and none indicated that the spines were flexible. Three new specimens are now known. Two consist of parts and counterparts (USNM 533989 and 533990; Figs. 1-2); they preserve the anterior valves and spines (Pojeta and DuFoe 2006) and show that the hollow spines were flexible. The third specimen (USNM 533991) is fragmentary; it preserves three valves in oblique cross section and partial impressions of a pair of spines and is not figured. Herein, the 2003 reconstruction of *E. dufoei* is shown, as is the new reconstruction.

Repositories: The specimens figured herein are reposited at the Department of Paleobiology, United States National Museum of Natural History (USNM), Washington, D.C. or at the Burpee Museum of Natural History (BMNH), Rockford, Illinois, U.S.A.

MATERIALS AND METHODS

All seven known "crack-out" specimens of *Echinochiton dufoei* were collected from a 7-15 cm thick mollusc-rich bed of dolostone near the top of Bauer's Quarry west of Beloit, Wisconsin, in the Forreston Member of the Grand Detour Formation, Platteville Group, of Middle Ordovician (Turinian; Blackriveran) age (Hoare and Pojeta 2006). Catalani and Frey (1998) noted that the Forreston Member was deposited in a tropical, shallow-water, carbonate platform environment. Kolata (1975: 11) wrote that studies of the Platteville Group and the lower part of the overlying Galena Group "strongly suggest an open platform, shallow to deep subtidal, normal marine environment. . . ."

Most fossils in the 7-15 cm bed are found parallel to bedding and occur in "pockets of accumulation." Cephalopods and pelecypods are the most abundant and diverse molluscs in the bed; 44% of the 25 known genera of cephalopods in the Forreston Member occur in this thin bed (DuFoe *et al.* 2006). Pelecypods are well represented by several species of palaeotaxodonts and pteriomorphians. Gastropods and bellerophonts are less abundant. Rostroconchs are known from a few specimens of *Eopteria* Billings, 1865. To date, chitons are the only group that has been studied in detail (Pojeta *et al.* 2003, Hoare and Pojeta 2006); this class is represented by three species, none of which is abundant. All of the molluscs are preserved as molds and casts.

The non-molluscan fauna includes strophomenoid brachiopods, bumastid trilobites, ostracodes, and fragmentary bryozoans and corals usually having well-preserved exoskeletons.

INTERPRETATION OF THE SHELL BED

The 7-15 cm shell bed is a death assemblage or *thanatocoenosis*. The fossils occur in "pockets of accumulation" separated by areas with few or no shells. The pockets indicate an irregular sea bottom with the shells accumulating in the low areas.

That the shells have been moved to their present location is likely because most valves of the pelecypods are disarticulated, as are the valves of the few brachiopods. Except *Echinochiton dufoei*, the chiton remains are disarticulated

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* From the symposium "Advances in Chiton Research" presented at the joint meeting of the American Malacological Society and Western Society of Malacologists, held 29 July to 3 August 2006 in Seattle, Washington.

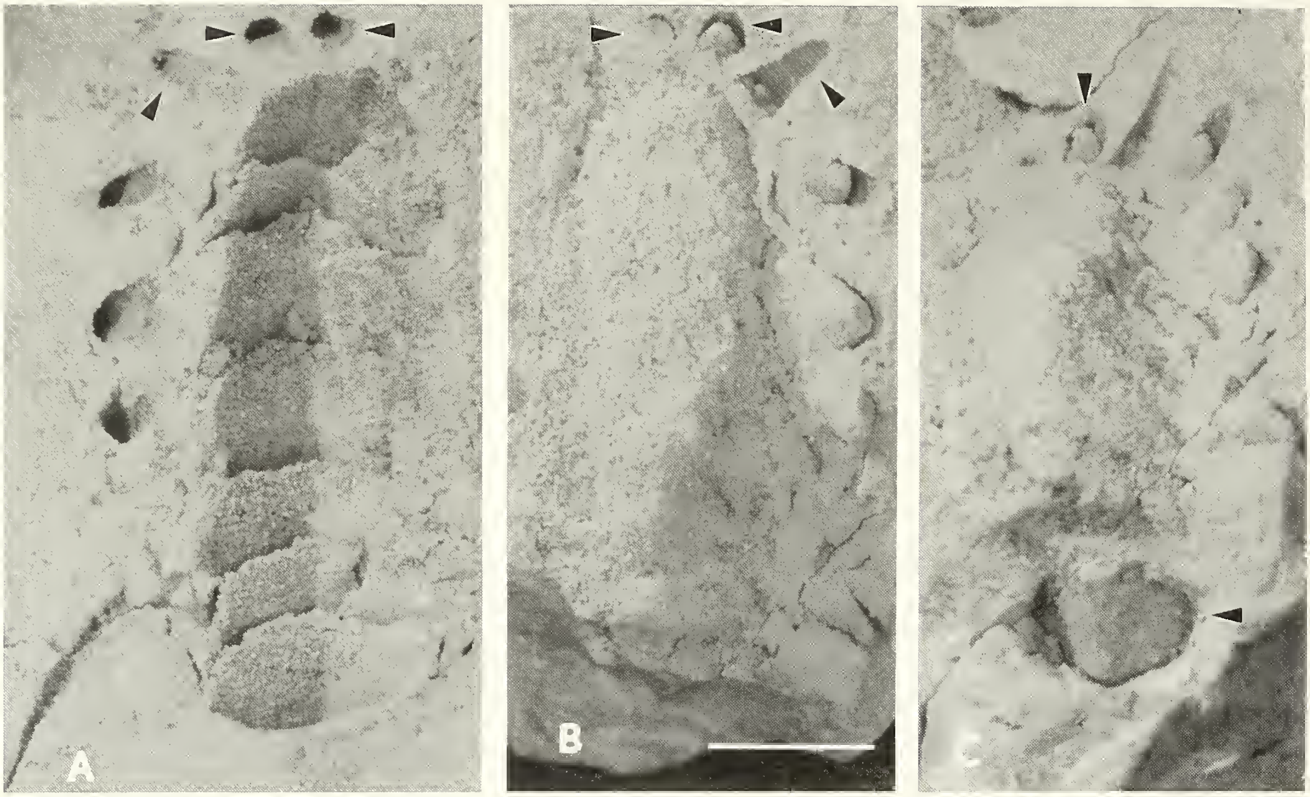

Figure 1. *Echinochiton dufoei* topotype (USNM 533989), anterior end up; scale bar 5 mm applies to all three views. A, Part, showing the anteriorly rounded head valve and valves 2-7 as internal molds of their ventral sides. Oblique arrow points to external mold of the morphologically right-lateral head valve spine. Horizontal arrows point to holes left by anterior head valve spines; these spine impressions are vertical to bedding. B, Counterpart filling of the body space below the valves. Arrows point to the same features seen in A; anterior spine impressions are filled with sediment. C, Counterpart tilted away from observer in order to show the tail valve (horizontal arrow), which is preserved at right angles to bedding. Vertical arrow marks the right-lateral sediment-filled anterior spine of the head valve.

plates. Most of the cephalopods are preserved as short fragments of phragmocone attached to short sections of the living chamber. The gastropods have abraded apertures. The trilobites are usually disarticulated into their constituent parts, and the corals and bryozoans are fragmented. That the assemblage was not moved far is indicated by some of the pelecypods that remained articulated or are preserved with the two valves splayed open and lying side by side (butterflyed) parallel to bedding and with the umbos touching.

Echinochiton dufoei is an exception to the general preservation of the other fauna, in that all known specimens are partially articulated although disarticulated spines are known.

THE NEW SPECIMENS

As in previous specimens, the new material is preserved as complex molds. In specimen USNM 533989 (Figs. 1A-C) the counterpart (Figs. 1B-C) shows impressions, or sedimentary fillings, of five of the hollow spines on the right side, including a right-lateral spine on the head valve, and two spines at the anterior end of the head valve. The sedimentary filling of the body space does not show the valves well. The part of the specimen (Fig. 1A) shows the undersides of the anteriorly rounded head valve and valves 2-7 parallel to bedding. The tail valve is at right angles to bedding and is preserved on the counterpart (Figs. 1B-C). The external mold of the right-lateral spine on the head valve is preserved as an impression. Anterior to the head valve are the impressions and fillings of two spines (Figs. 1A-B).

The length of the eight valves is about 25 mm, the width of a single intermediate valve is 6 mm, and the length of the one complete and curved spine preserved parallel to bedding on the right side of valve 6 (Fig. 1A) of the part is 7.5 mm.

It is noteworthy that the impressions of the spines, or their sedimentary fillings, of this specimen are preserved at various angles to bedding, ranging from parallel to right angles (Figs. 1A-B), thus indicating that the spines were

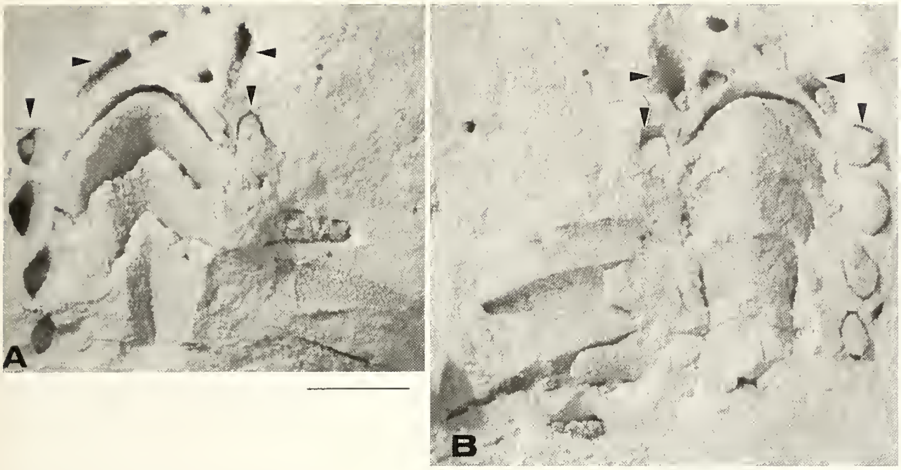

Figure 2. *Echinochiton dufoei* topotype (USNM 533990), anterior end up; scale bar 5 mm applies to both views. A, Part, showing the anteriorly rounded head valve and valves 2 and 3 as internal molds of their ventral sides. The lateral head valve spines (vertical arrows) are preserved vertical to bedding as are the spines on the morphologically right side. The spines on the morphologically left side of valves 2 and 3 are preserved parallel to bedding. The anterior head valve spines are preserved rotated counterclockwise to the morphologically left side and have most of their lengths are exposed (horizontal arrows). B, Counterpart filling of the body space below the valves showing the anteriorly rounded head valve. Arrows mark the same features as in A above. Left-lateral external molds of spines partially filled with sediment.

flexible. All previously known specimens of *Echinochiton dufoei* have the hollow spines preserved parallel to bedding (Pojeta *et al.* 2003, figs. 1-6). As used herein, the word flexible means that at least after death the spines were capable of being bent. Specimen USNM 533990 (Figs. 2A-B), part and counterpart, preserves the anteriorly rounded head valve, valves 2-3 on the part (Fig. 2A), and the head valve and valves 2-4 on the counterpart (Fig. 2B). On the left side of the counterpart, the hollow spines attached to valves 2-4 are preserved parallel to bedding and show some of the sediment fillings of the bases of the spines. On the right side, the sediment fillings of the spines of valves 2-4 are preserved at right angles to bedding (Fig. 2B). The lateral spines of the head valve are preserved at right angles to bedding. The anterior spines of the head valve have been rotated counterclockwise to the left (Fig. 2B); they are at a low angle to bedding and much of their lengths are exposed. This specimen also shows that the spines were flexible, because they are preserved at various angles to bedding.

The length of the four preserved valves is 14 mm (valve 4 is incomplete), the width of valve 2 is 6 mm, and the length of the spine on the left side of valve 4 (Fig. 2B) is about 11 mm (attachment to valve 4 not visible).

Neither of the new specimens described here show well-preserved scutes or the slots made by scutes in internal molds; a few slots are on the right side (left side in view, Fig. 1A). The scutes are erect structures that occur in right and left rows between and parallel to the valves and between the valves and the hollow spines (Figs. 3A, 3B). The sediment-filled hollow spines are best preserved on the holotype part (BMNH 1996.045.01) and counterpart (BMNH 1996.045.02) and are parallel to bedding (Figs. 3A, 4).

See Figure 5 for the new reconstruction and the 2003 reconstruction.

MOVEABLE SPINES?

So far as is known, *Echinochiton dufoei* is unique among chitons in its possession of circumsomal hollow spines that are as long, or longer, than the valves are wide (Figs. 1-5). Our search of the literature yielded no other chitons with the large, hollow spines of *E. dufoei*. Eernisse (e-mail comm., September 2006), commenting on *E. dufoei*, noted: "There are no other hollow spines documented [in chitons] that I know of. We (with Pat Reynolds) cite studies in my chapter on chitons: Eernisse, D. J., and P. D. Reynolds, 1994."

Scheltema *et al.* (1994: 20) noted the existence of solid and hollow spicules in the epidermis of neomenioid aplacophorans. However, these are small structures, about 20-200 microns long "and are secreted extracellularly within an invagination of a single cell." Thus, it is highly unlikely that the hollow spicules of aplacophorans are homologous with the hollow spines of *Echinochiton dufoei.*

The new specimens show that the spines were flexible. However, were they moveable in life, or is the flexibility a post-mortem effect? Examine the color photographs of the holotype part and counterpart before the specimen was prepared and before it was whitened with ammonium chloride sublimate (Fig. 6); compare this with the prepared specimen (Figs. 3A and 4). It is noteworthy that the impressions of the spines are much darker than the surrounding rock and the valves; this is also the case with some of the spines (Fig. 2) and the specimens in Pojeta *et al.* (2003: figs. 5.2, 6.3, 6.4).

This darker color suggests that the spines contained more organic matter than did the valves. In some Ordovician mytiliform and modioliform pelecypods, the internal molds and the ligament area are covered with a black film (Pojeta 1962: 175; Pojeta 1971: pl. 15, figs. 5, 6). This

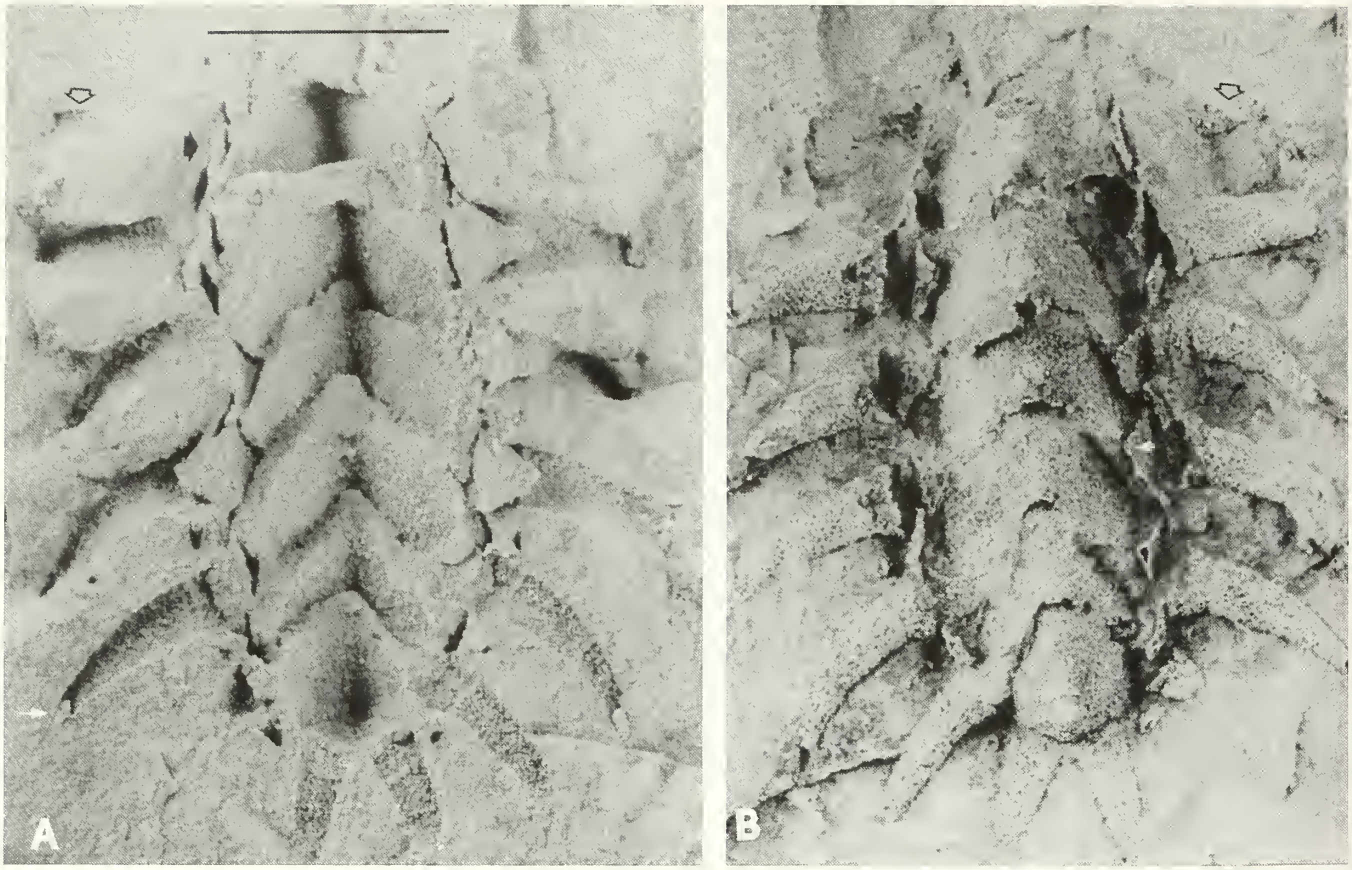

Figure 3. *Echinochiton dufoei* holotype (BMNH 1996.045.01), anterior end up; scale bar 10 mm applies to both views. A, Part, showing valves 3-8 as internal molds of their ventral sides, mucro of tail valve, external mold impressions of spines parallel to bedding, posterior spines of tail valve, partial sediment fillings of hollow spines, and slots paralleling the right and left lateral side of the valves (solid black arrow). Open black arrow points to a remnant impression of the morphologically right side spine of valve 3. White arrow at the tip of the external mold of the morphologically right side spine of valve 7 shows that the sediment filling of the spines went to their distal ends; thus, the spines were hollow to their tips. B, Latex cast of A, white arrow points to one of the scutes on the right side. Open black arrow at top right marks the same structure as in A.

presumed organic film does not extend into the surrounding sediment; it is limited to the internal mold. The ligament and the thick periostracum of extant mytiliform and modioliform pelecypods contains more organic material than in other areas of the shell and in other groups of pelecypods. This possible larger organic component of the spines in *Echinochiton dufoei* may help explain the flexibility of the spines.

The part of the holotype (Fig. 3A) preserves external impressions of the hollow spines, the underside of the valves, and rows of slots between the valves and the spines. The latex positive (Fig. 3B) shows that the slots are molds of right and left rows of raised triangular scutes between the valves and the spines. The scutes can occur between the spines or between the spines and the valves.

The spines show growth lines; thus, it seems likely that mantle extended into them at least to the bases of the spines (Fig. 7A). If this tissue had sufficient muscle fibers, it could have moved the spines with the scute acting as a fulcrum against which to raise and lower them cantilever style or to move the spines fore and aft in a rotary fashion. The scutes bend toward the valves and together with the spine could form a ball-and-socket joint, analogous to what is seen in regular echinoids in which the solid spine is mounted on a tubercle (Hyman 1955, fig. 187A). In echinoids, the outer cylinder of muscle fibers, by local contraction, causes the spine to point in the direction of the stimulus (Hyman 1955: 438).

In both our old and new reconstructions (Fig. 5), we show the spines as being embedded in the mantle girdle. It is unlikely that they would be below the girdle, because this would impede the ability of the animal to attach to the

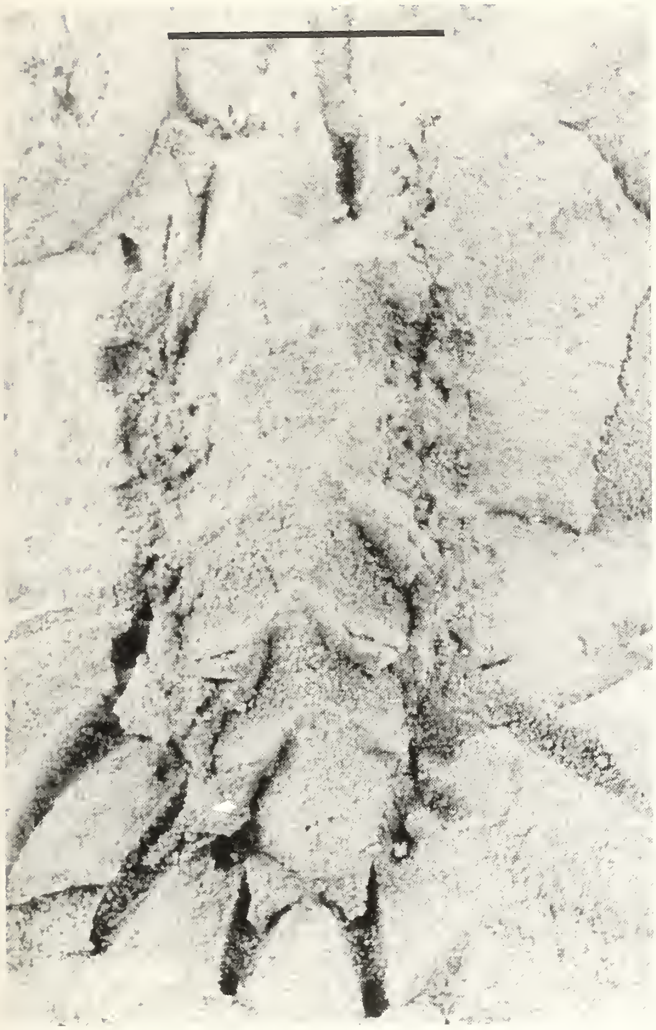

Figure 4. *Echinochiton dufoei* holotype (BMNH 1996.045.02), anterior end up; scale bar 10 mm. Counterpart filling of the body space below the valves of Fig. 3A. White arrow on the left lateral spine of valve 7 shows that the lumen of the spine decreased in size distally because the sediment filling decreases in size.

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substrate. If the spines were embedded in the girdle, vertical movements would probably be limited and movement would be mostly in rotary anterior and posterior directions. Thus, the spines preserved at right angles to bedding would be largely a post-mortem effect. It is unlikely the spines were

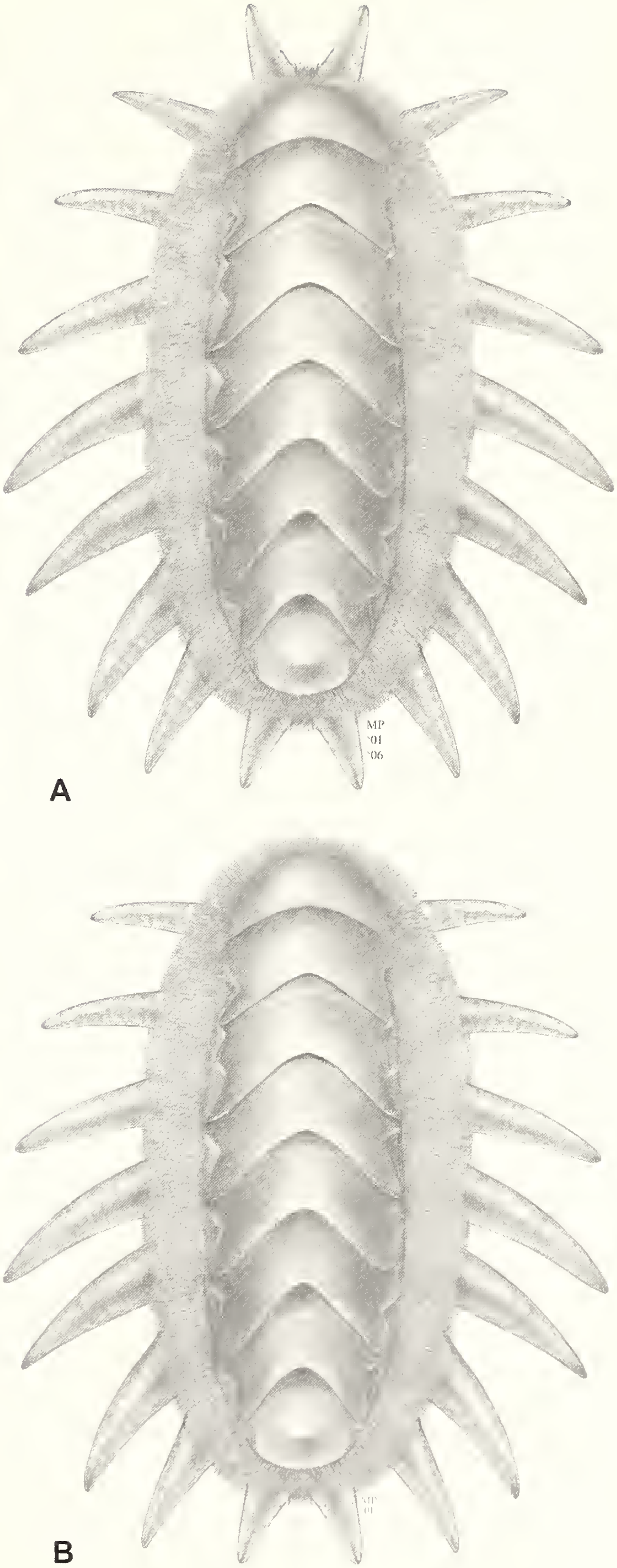

Figure 5. *Echinochiton dufoei.* A, New reconstruction incorporating the new information from specimens seen in Figs. 1 and 2. Anterior end up. B, 2003 (Pojeta *et al.*) reconstruction showing the lack of information about the anterior end of the species. The lateral spines on the head valve were postulated, but not seen, in 2003. Size about 2.0×.

above the girdle, because there seems to be no way that they could have attached to the shell in this position and have mantle extend into them. The presence of spicules and scales in the girdle is indicated by small horizontal markings at nearly right angles to the lateral edges of the valves (Pojeta *et al.* 2003: figs. 6.1 and 7.2; Figs. 7B, 8 herein).

WHY LARGE HOLLOW SPINES?

What function could the large hollow spines serve? Various reasons can be postulated although none of the hypotheses stand out as the most likely reason for such spines:

(1) Somehow the spines were used for protection from predation. However, all known specimens of *Echinochiton dufoei* are small, in the 25-40 mm range. The major predators in Ordovician time were shelled cephalopods, many of which were considerably larger than *E. dufoei.* The cephalopods found in the same bed as *E. dufoei* range in size from a few centimeters to over three meters, and *E. dufoei* would not even be a small snack for most of the cephalopods except young juveniles. In addition, hollow spines, even if made turgid with fluid, would provide little protection from predation. Thus, it seems unlikely that the spines were used for protection. Also, as suggested above, because the spines were probably embedded in the girdle, their vertical movement would be limited and most movement would be in oar-like anterior and posterior directions.

K. M. Brown (Louisiana State University) suggested that "the horizontal spines would make it harder to pry the animal from the substrate" (pers. comm., June 2007)

Various non-chiton molluscs have spines, including spondylid and some venerid pelecypods, some muricid gastropods, and a few shelled cephalopods. These spines may or may not have a lumen, or a longitudinal groove. All are strongly calcareous and range in shape from sharply pointed to spatulate. Generally these are regarded as protective or supportive in function.

The spines of productoid brachiopods are hollow, hard, and not flexible when fully formed. Muir-Wood and Cooper (1960: 16) regarded the spines as: "Forming a prominent part of the ornament and occur to some extent in every productoid species. The spines were in part protective, but

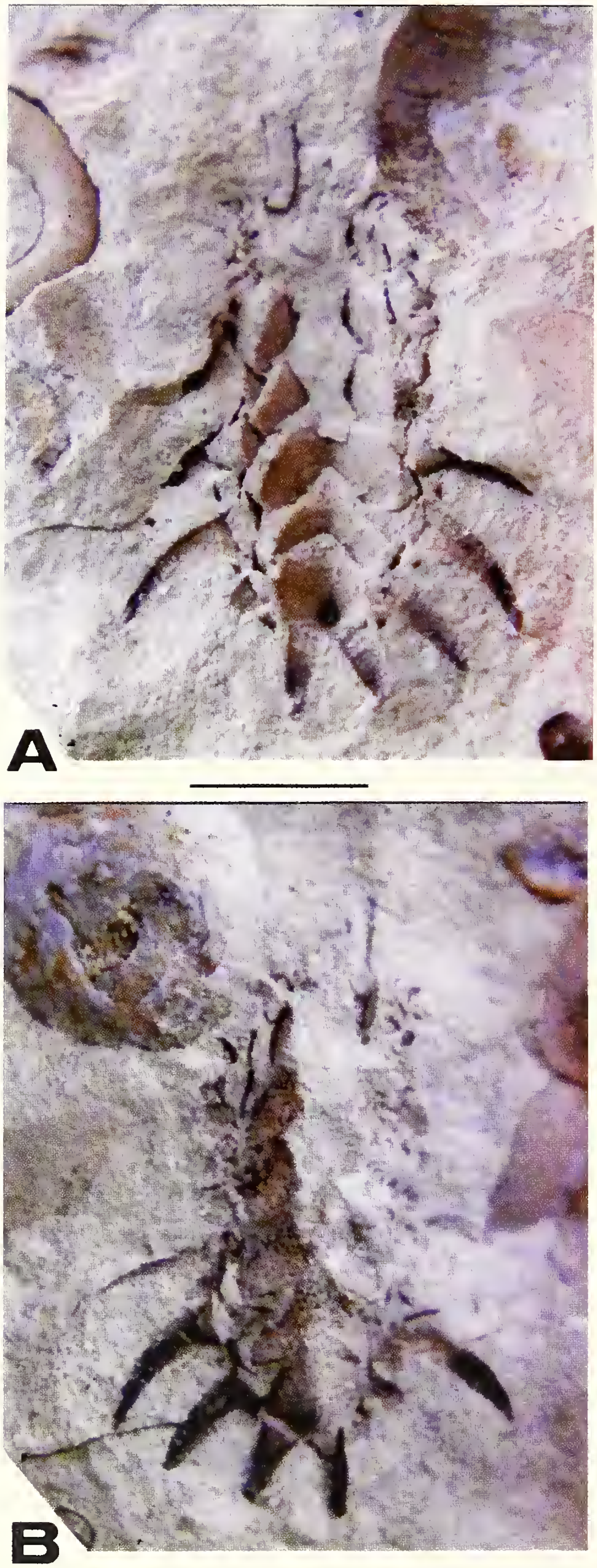

Figure 6. *Echinochiton dufoei*, anterior end up; scale bar 10 mm applies to both images. A, B, Color photographs of part and counterpart of holotype (BMNH 1996.045) as the specimen was found in the field and before it was prepared. Compare to Fig. 3A and 4. Note that many of the spine impressions are much darker than the impressions of the valves and the surrounding sediment.

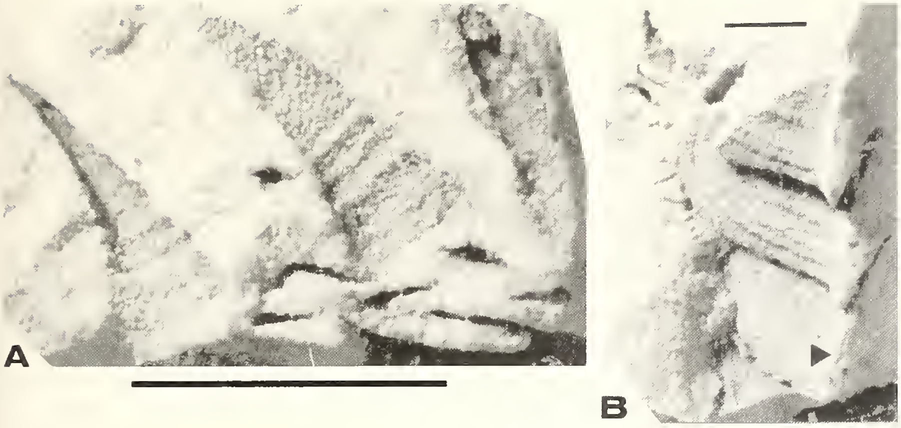

Figure 7. *Echinochiton dufoei* paratype (USNM 517481). A, Anterior to right; scale bar 10 mm. Enlargement of three lateral spine external molds showing growth lines. B, Anterior down; scale bar 10 mm. Underside of first three valves seen on the right side of Fig. 8, showing growth rugae. Lateral to the upper two valves, on the left side of the image, are marginal markings interpreted to be the impressions of scales and spicules. Black tailless arrow marks the same position as the black tailless arrow in Fig. 8.

they also played an important role in the attachment and support of the shell, and some may have functioned as strainers." Grant (1966) showed the support function of the multitudinous spines of the productoid *Waagenoconcha abichi* in soft sediments.

(2) David Pawson, USNM (pers. comm., September 2006) has observed that, among other functions, some regular echinoids use their spines to define their living space and form a checkerboard-like pattern on the sea floor. This seems unlikely for *Echinochiton dufoei* because it is such a rare element in the fauna of the 7-15 cm thick bed; in 15 years of collecting and breaking literally tons of rock from the 7-15 cm thick bed, only seven specimens have been found. Of course, *E. dufoei* may be found to be abundant elsewhere. However, the Ordovician fossils of Wisconsin and Illinois have been studied for at least 140 years, and the first mention of *Echinochiton* in the literature is Pojeta *et al.* (2003).

(3) The function of the spines is as stabilizers—something akin to outriggers when the animal moved. Alternatively, the spines may have helped to maintain the chiton's position on a hard substrate in strong waves and currents. However, extant chitons hug the substrate tenaciously using the foot and mantle girdle and are difficult to dislodge without using an instrument having a blade.

(4) The spines were somehow used as accessory organs of locomotion, particularly if their motion was largely in the rotary anterior-posterior directions.

TAXONOMIC PLACEMENT

Echinochiton dufoei is a polyplacophoran mollusc because of the row of eight bilaterally symmetrical valves (plates), the mucro on the tail valve, and the likely presence of a mantle girdle interpreted from small impressions lateral to the valves indicating the presence of spicules and scales. It differs from other polyplacophorans in the presence of circumsomal, large, hollow spines, and the right and left rows of scutes paralleling the outer edges of the valves. Thus, it is placed in the separate family Echinochitonidae.

Within the Polyplacophora, *Echinochiton dufoei* is in the subclass Paleoloricata based on the upright valves with large apical areas (Fig. 8); none of the known specimens show sutural laminae and they lack insertion plates. It is treated as a member of the order Chelodida because the intermediate valves are not clearly differentiated into lateral and central areas (Figs. 1A, 2B)

When commenting on *Echinochiton dufoei*, Sirenko (2006: 33) misunderstood Pojeta *et al.* (2003). His thought that the specimens were external molds is only partially correct. The specimens are very complex molds; the only part of the molds that are clearly external are the impressions of the outside of the spines when parallel to bedding (Fig. 3A). The sedimentary fillings of the spines are internal molds (Fig. 4). The specimens are internal molds of the ventral sides of the valves (Figs. 1-4).

To date, most of the external molds of the dorsal side of the valves have been found in a specimen where they cannot be seen (Fig. 8). An exception to this is the partial impression of the external surface of the head valve (Fig. 1A); it does not preserve the external features.

Sirenko's notation that *Echinochiton dufoei* has small apical areas is incorrect as shown herein by the specimen (Fig. 8) in which the valves are seen in lateral view. The valves are nearly erect and have large apical areas. The spaces between the internal and external molds represent a minimum thickness for the valves. In this specimen, the external molds of the valves could not be exposed.

The valves that are preserved parallel to bedding so that they cannot be seen in lateral view may have been distorted by compaction (compare the shapes of the valves in Figs. 1A, 2A, and 3A). Also when comparing the shapes of the counterparts (Figs. 2B, 3B, 4), there is variation in the shapes of the valves.

In *Echinochiton dufoei*, Sirenko suggested the presence of incisura; it is not clear to us what the term incisura means.

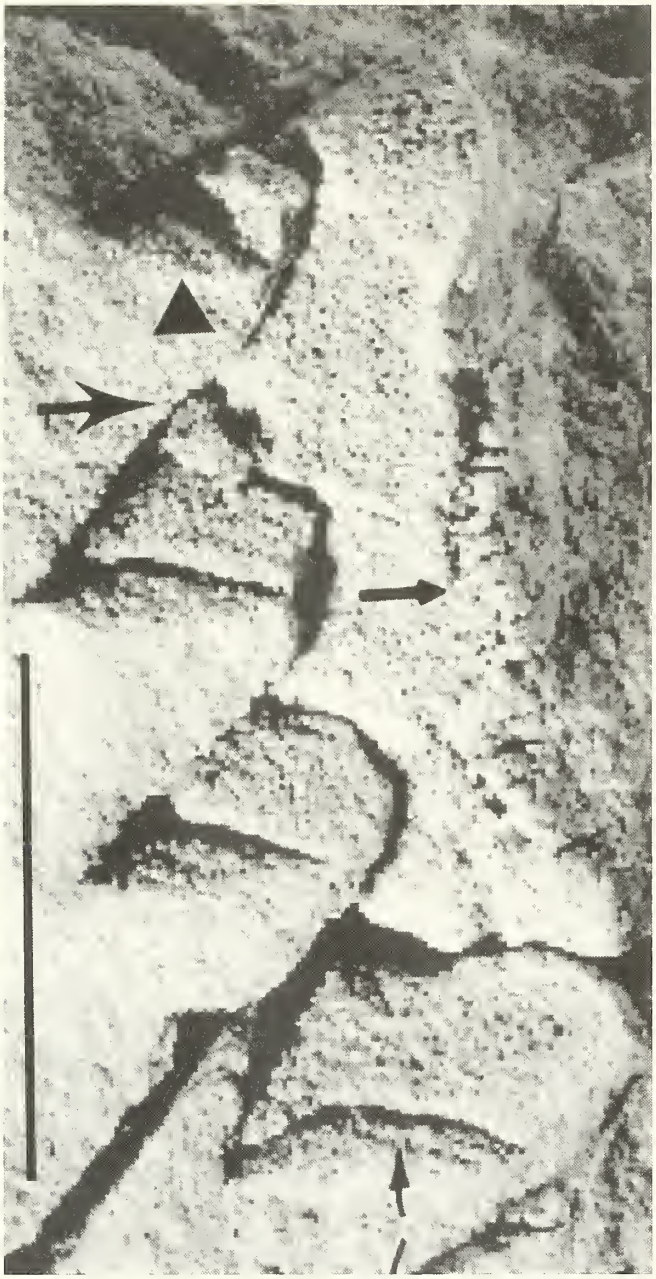

Figure 8. *Echinochiton dufoei* paratype (USNM 517481), anterior end to right; scale bar 10 mm. Lateral view of four valves showing their nearly erect posture and the filling of the body space below the valves. Downward facing straight-tailed barbed arrow points to two sediment fillings of the valve suggesting the presence of two openings into the valve as in *Matthevia variabilis* Walcott, 1885. Downward facing straight-tailed unbarbed arrow points to markings lateral to the body that are interpreted as having been made by spicules in the girdle. Wavy arrow at left end points to a piece of a fifth valve that is only partially preserved. Solid triangular tailless arrow is at the same position as in Fig. 7B.

Hyman (1967: 74) used the term incisures as follows: "The insertion plates are commonly cut into teeth by incisures . . . These incisures are continued . . . as slit grooves known as slit rays." *Echinochiton dufoei* does not show insertion plates and, thus, lacks slit rays separating the insertion teeth from the rest of the shell (Fig. 1A).

PHYLOGENETIC RELATIONSHIPS

Echinochiton shows similarities to mattheviids which are the oldest known chitons. In *Matthevia* Walcott, 1885, the valves are nearly upright and they have large apical areas (Runnegar *et al.* 1979, Vendrasco *et al.* 2004) as does *Echinochiton* (Fig. 8). The suggestion of a relationship between *Echinochiton dufoei* and *Matthevia* is reinforced by a plate of *E. dufoei* which shows the filling of two holes in the valve (Fig. 8) as is the case in *Matthevia variabilis* Walcott, 1885, the type species of *Matthevia.* Hoare (2000) placed mattheviids at the base of his phylogenetic scheme of polyplacophorans.

Caron *et al.* (2006) discussed and redefined the large, Middle Cambrian, Burgess Shale species *Odontogriphus omalus* Conway Morris, 1976. This animal approximates a shell-less polyplacophoran. Caron *et al.* (2006) noted that the species is up to 125 mm long, is flattened dorsoventrally, and has a dorsal stiffened cuticle, a radula, a straight gut, and simple gills that are present in a groove running laterally and posteriorly around a muscular foot. Stratigraphically, *Odontogriphus* Conway Morris is older than the first known chitons which occur in Upper Cambrian rocks (Vendrasco and Runnegar 2004). At the very least, an animal closely resembling *Odonogriphus* would be a likely stem group for chitons.

Echinochiton increases the known disparity of polyplacophorans. Vendrasco *et al.* (2004) considered Paleozoic chiton disparity to be even greater than that suggested by *Echinochiton*, after the discovery of a nearly complete Early Mississippian specimen of the taxon *Multiplacophora* Hoare and Mapes, 1995.

The new species *Polysacos vickersianum* Vendrasco *et al.*, 2004, is about 25 mm long. The shell has three longitudinal columns of valves plus head and tail valves, and it is surrounded by many elongate hollow spines. The authors noted the similarity of this arrangement to *Echinochiton dufoei* in which the skeleton has a central column of valves, two flanking columns of small dorsally projecting scutes, and is surrounded by hollow spines that grew by accretion. In both groups the anterior and posterior valves are morphologically distinct from the intermediate valves. Vendrasco *et al.* (2004) regarded the skeleton of *E. dufoei* as intermediate in form between the skeletons of multiplacophorans and typical chitons. As in "crown group chitons", multiplacophorans have pores in valve surfaces and possess the articulamentum (inner shell layer) that projects from the growing margin of the shell. *Echinochiton* and other stem group chitons are not known to have the articulamentum.

As noted by Vendrasco *et al.* (2004), there are significant differences between chitons and multiplacophorans such as *Polysacos*. The most striking difference is that in *Polysacos* there is a seven-fold, rather than an eight-fold iteration of the major skeletal elements.

The cladistic analysis performed by Vendrasco *et al.* (2004: 288) "Strongly supports the placement of multiplacophorans with the total group Polyplacophora." They treat the Multiplacophora as an order within the class Polyplacophora.

Another recently-discovered Paleozoic multi-plated species is *Acaenoplax hayae* Sutton *et al.*, 2001. Sutton *et al.* (2004) monographed the species. The species is known from Middle Silurian age rocks. This species is vermiform, up to 40 mm long, and has one ventral and seven dorsal valves. The dorsal valves are not articulated and most are separated by a variable number of dorsal ridges. Most of the ridges bear elongate, thin, rigid, and pointed spines, and although it is not noted if the spines were hollow, this seems unlikely. Sutton *et al.* (2001a) regarded *Acaenoplax* Sutton *et al.*, 2001 as a mollusc, perhaps allied to the Aplacophora and showing some polyplacophoran affinities.

Subsequent to Sutton *et al.* (2001a), there was a debate about the treatment of *Acaenoplax* as a mollusc. Steiner and Salvini-Plawen (2001) argued that *Acaenoplax hayae* was best considered to be allied to polychaete annelids; Sutton *et al.* (2001b, 2004) defended the molluscan affinities.

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Methods of sample preparation of radula epithelial tissue in chitons (Mollusca: Polyplacophora)*

Jeremy A. Shaw[1], David J. Macey[1], Peta L. Clode[2], Lesley R. Brooker[3], Richard I. Webb[4], Edward J. Stockdale[2], and Rachel M. Binks[1]

[1] School of Biological Sciences and Biotechnology, Murdoch University, Murdoch, Western Australia, 6150, Australia, jeremy.shaw@uwa.edu.au

[2] Centre for Microscopy, Characterisation and Analysis, The University of Western Australia, Crawley, Western Australia, 6009, Australia.

[3] Faculty of Science, Health and Education, University of the Sunshine Coast, Maroochydore, District of Cairns, Queensland, 4558, Australia.

[4] Centre for Microscopy and Microanalysis, Department for Microbiology and Parasitology, University of Queensland, St. Lucia, Queensland, 4072, Australia.

Abstract: A glutaraldehyde fixative developed for preserving the radula superior epithelium of the adult chiton *Acanthopleura hirtosa* (Blainville, 1825), was used in conjunction with conventional and microwave-assisted sample processing to produce high quality tissue preservation for light and electron microscopy. In addition, high-pressure freezing (HPF) and cryo-substitution were used to fix the radula tissue of juvenile specimens. Microwave-assisted fixation was preferred to conventional bench-top techniques due to the superior preservation of fine cell structure together with reduced processing times and chemical exposure. Although restricted to very small (<200 μm) samples, the quality of juvenile radulae processed by HPF was excellent. The improvements in tissue preservation using microwave and cryo-preservation techniques are therefore critical for obtaining accurate ultrastructural information on the radula in marine molluscs. In particular, these findings highlight additional processing options available for the study of cellular structures in biomineralizing tissues.

Key words: microwave, high-pressure freezing, chemical fixation, cryo-fixation, biomineralization

The radula has been the focus of numerous studies over many decades, with its intricate and varied design used to elucidate aspects of molluscan ecology, biology, and taxonomy (Fretter and Graham 1962, Runham 1963, Steneck and Watling 1982, Padilla 1985, Scheltema 1988, Salvini-Plawen 1990). In addition, the radulae of polyplacophoran and patellid gastropods have received particular attention as a result of their unique ability to harden their teeth with iron and other biominerals (Mann *et al.* 1986, Lowenstam and Weiner 1989, Webb *et al.* 1989).

The chiton radula represents an excellent example of matrix-mediated biomineralization, where minerals are formed in a highly organized manner within the framework of an organic matrix (Simkiss and Wilbur 1989, Watabe 1990, Mann 2001, Weiner and Addadi 2002). While considerable progress has been made in elucidating the general structural organization of minerals deposited within the tooth matrix and the sequence in which they are deposited (Lowenstam 1962, Kim *et al.* 1989, Macey *et al.* 1994, Brooker *et al.* 2006), the mechanisms involved in cellular transport of ions to the tooth cusps are poorly understood. The elemental precursors for biomineralization are thought to be delivered to the teeth by the overlying, superior epithelial tissue, which surrounds the cusps during all stages of development (Nesson and Lowenstam 1985) (Fig. 1). The superior epithelium and teeth are encapsulated within the radular sheath, the inferior epithelium, and the radula membrane with the whole structure resembling a tube open only along the dorsal surface (Fig. 1). Histological investigation of epithelial tissue is difficult due to the complex composition and structure of the radula, which contains both hard mineralized structures and cartilaginous membranes in close proximity to cellular material.

Chemical fixatives such as glutaraldehyde buffered in filtered seawater are commonly used for preserving marine organisms, where the osmotic pressure of the solution acts to mimic that of the animal, thereby reducing swelling or shrinkage of the tissues (Dykstra and Reuss 2003). However, fixation often gives rise to variations in the ultrastructural morphology of organelles (Hayat 2000), and it is therefore preferable to utilize several techniques to acquire comparative information. Microwave-assisted fixation and cryo-preservation techniques (McDonald *et al.* 2007, Webster 2007), now common in laboratories, provide two additional means of obtaining such comparative information. The main advantage of microwave protocols is a dramatic reduction in sample processing times (<4 hours), while at least maintaining, if not improving, fixation quality over conventional methods (Giberson and Demaree 1999, Laboux *et al.* 2004). Excellent tissue preservation can also be attained using cryo-preservation techniques, which achieve vitreous ice formation in samples and thereby prevent ice crystal damage. However, due to difficulties associated with heat dissipation, only very small samples (<200 μm) can be frozen successfully (Wilson *et al.* 1998, Sawaguchi *et al.* 2005).

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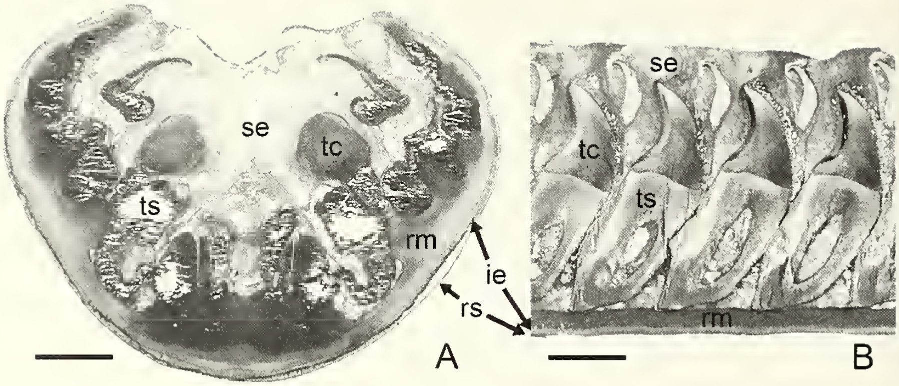

Figure 1. Radula apparatus of the chiton *Acanthopleura hirtosa* (adult) in (A) transverse section and (B) longitudinal section exhibiting various hard and soft tissue components. Abbreviations: se, superior epithelium; ie, inferior epithelium; tc, tooth cusp; ts, tooth stylus; rm, radula membrane; rs, radula sheath. Scale bars = 200 μm.

To improve our current techniques and better understand the detailed cellular structure of the superior epithelium in chiton and limpet teeth, we investigated alternative fixation methods. Determining the precise structure of these cells will assist in elucidating their function and the mechanisms involved in the transport of ions into the teeth, a fundamental obstacle to our wider understanding of the initial phase of biomineralization. The aim of this study is to compare three fixation methodologies (conventional chemical, microwave-assisted, and low temperature), regarding preservation of the superior epithelial tissues of the chiton *Acanthopleura hirtosa* (Blainville, 1825) for both light and electron microscopy.

MATERIALS AND METHODS

Conventional and microwave-assisted chemical fixation and cryo-fixation techniques were utilized for the preparation of epithelial tissues for light microscopy (LM) or transmission electron microscopy (TEM). Adult and juvenile specimens of the chiton *Acanthopleura hirtosa* (mean animal length ~4 cm and ~0.8 cm, respectively) were collected from intertidal limestone at Woodman Point within the Perth metropolitan area of Western Australia (32°08′S, 115°44′E). Incisions were made along both pallial grooves from the anus towards the head, thereby freeing the foot, visceral mass, and buccal mass as a single entity from the shell plates and girdle. The visceral mass was then carefully removed to expose the radula sac. Dissections were performed as quickly as possible to reduce the deterioration of radula epithelial tissue.

Table 1. Conventional and microwave-assisted processing schedules for the radula epithelial tissues of adult *Acanthopleura hirtosa.*

Step	Medium	Concentration (%)	Conventional times	Microwave times	Microwave wattage (W)
Fixation	glutaraldehyde (buffer A)	2.5*	24	2×($2^{on}/2^{off}/2^{on}$)(min)(v)	80
Rinse	buffer A		15 min ×4	40 sec (v)	250
Post-fixation	OsO_4 (buffer B)	1*	2 h	2×($2^{on}/2^{off}/2^{on}$)(min)(v)	80
Rinse	buffer B		15 min ×4	40 sec (v)	250
Dehydration	acetone	10, 30, **50***, **75***, **90***, **100***	15 min ×2 each	40 sec each (100 ×2)	250
Infiltration	Spurr's resin	5, 10, 20, 40, **50**, 60, **75**, 80, **90**, **100***	8-12 h each	3 min each (100 ×2) (v)	250
Polymerization	Spurr's resin	**100***	conventional oven 60 °C overnight		

Note: Concentrations in bold represent the microwave schedule, concentrations not in bold represent the conventional schedule, and * represents concentrations used in both schedules.

(v) = steps undertaken in vacuum

$^{on/off}$ denotes magnetron (irradiation) cycle

Table 2. Conventional and microwave-assisted processing schedules for the radula epithelial tissues of juvenile *Acanthopleura hirtosa.*

Step	Medium	Concentration (%)	Conventional times	Microwave times	Microwave wattage (W)
Fixation	glutaraldehyde (buffer A)	2.5*	24 h	$2\times(2^{on}/2^{off}/2^{on})$ (min) (v)	80
Rinse	buffer A		10 min ×4	40 sec (v)	250
Post-fixation	OsO_4 (buffer B)	1*	1 h	$2\times(2^{on}/2^{off}/2^{on})$ (min) (v)	80
Rinse	buffer B		10 min ×4	40 sec (v)	250
Dehydration	ethanol/acetone$^{(a)}$	**50***, **75***, **90***, **100***, **100**$^{(a)}$, 100$^{(a)}$	10 min each (100 ×2)	40 sec each	250
Infiltration	Procure Araldite	5, 20, **50**, 60, **75**, 80, **90**, **100***	4-8 h each	3 min each (100 ×2) (v)	250
Polymerization	Procure Araldite	**100***	conventional oven 60 °C overnight		

Note: Concentrations in bold represent the microwave shecule, concentrations not in bold represent the conventional schedule, and * represents concentrations used in both schedules.

(v) = steps undertaken in vacuum

$^{on/off}$ denotes magnetron (irradiation) cycle

$^{(a)}$ = acetone

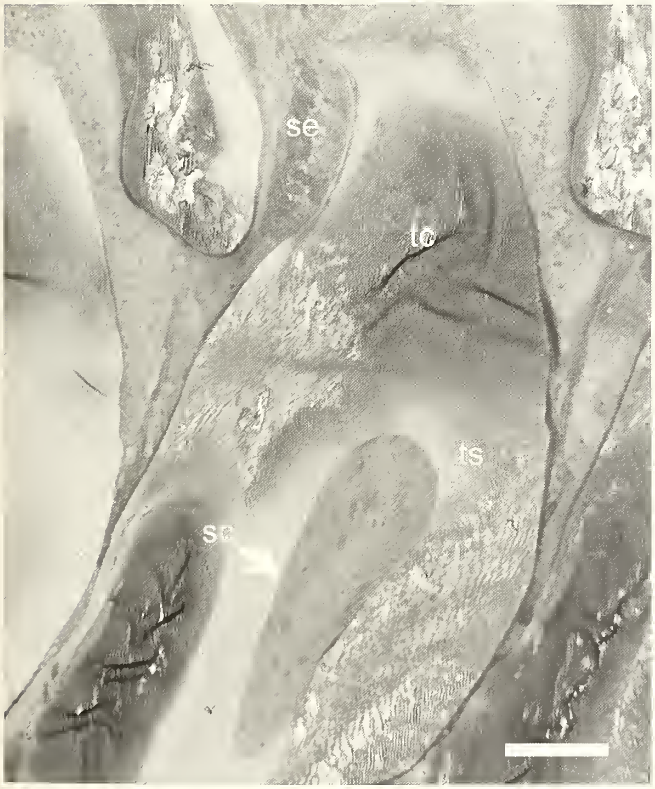

Figure 2. Light micrograph of a longitudinal section through a major lateral tooth from adult *Acanthopleura hirtosa* at row 6 prepared using microwave-assisted protocols. Despite being situated deep within the tooth stylus (ts), the tissues of the stylus canal (sc) are well preserved. se, superior epithelium; tc, tooth cusp. Scale bar = 50 µm.

Preparation of radulae from adult animals

For samples processed using either conventional or microwave-assisted methods, the dissected tissue mass was immediately immersed in a fixative comprised of 2.5% glutaraldehyde buffered in 0.1 M phosphate, with a pH of 7.2 and an osmolarity of 900 mmol kg^{-1} adjusted using sucrose (buffer A). The buccal mass and radula were separated from the remainder of the animal, and the radula was either left whole or cut transversely into three or four segments (the buccal mass was discarded). The tissues were then processed by either conventional bench-top methods or accelerated microwave-assisted protocols using a Pelco Biowave® fitted with a cold spot and vacuum chamber, according to the specific schedules detailed in Table 1. This included fixation with glutaraldehyde in buffer A, rinsing in buffer A, post fixation in 1% osmium tetroxide (OsO_4) in 0.05 M phosphate buffered saline (buffer B) at 4 °C, and a final rinse in buffer B, prior to dehydration through a graded series of acetones, then infiltration and embedding in Spurr's resin. Preparation of radula tissue by high-pressure freezing was not possible for adults due to limitations in sample size, which was again restricted to ~200 µm.

Following polymerization, resin blocks were sectioned for observation at both the LM and TEM level. For LM, 1 µm-thick sections were mounted on glass slides and stained with aqueous 1% Methylene Blue and 1% Azur II (20 sec) prior to imaging on an Olympus BX51 optical microscope fitted with an Olympus DP70 digital camera. For TEM, ~60 nm-thick sections were mounted on copper grids and double stained with uranyl nitrate (single crystal in one drop of 50% methanol) (10 min) followed by Sato's modified lead citrate (10 min) (Hanaichi *et al.* 1986) prior to imaging on a JEOL 2000 TEM at 80 kV.

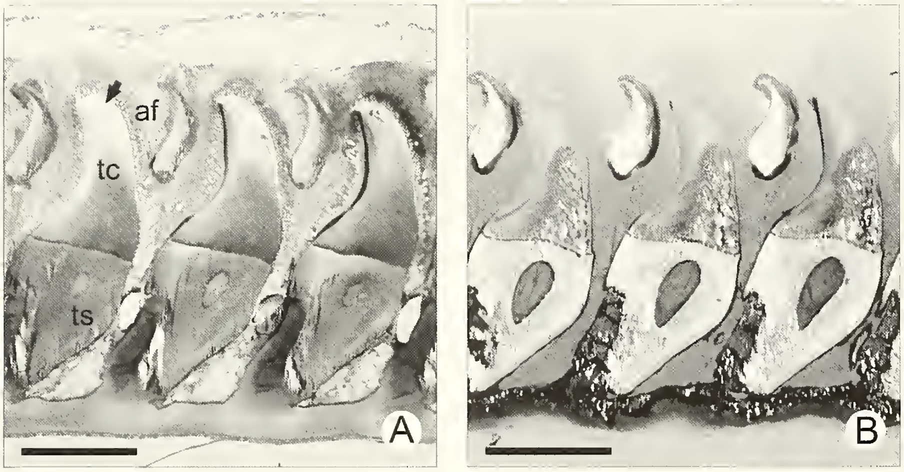

Figure 3. Major lateral tooth rows 12-14 from radulae of adult *Acanthopleura hirtosa* prepared using (A) conventional and (B) microwave techniques. Note the separation (arrow) of the superior epithelium from the anterior face (af) of the tooth cusps (tc) in conventionally fixed material and the poor infiltration of resin into the tooth stylus (ts) and cusp in microwave prepared material. Scale bars = 200 μm.

Preparation of radulae from juvenile animals

Juvenile specimens of *Acanthopleura hirtosa* were processed similarly using conventional and microwave-assisted methods; however, schedules were adjusted in order to account for the reduction in tissue size, and samples were embedded in Procure Araldite (formerly Epon Araldite) (Table 2). For cryo-fixation of juvenile radulae, the immature portion of each radula was removed by cutting transversely approx. 200 μm from the posterior end. Each immature portion was then placed into a 200 μm membrane filled with 20% bovine serum albumin in artificial seawater. These membrane-mounted samples were rapidly frozen in a high-pressure freezer (Leica EMPACT 2) prior to cryo-substitution in acetone containing 2% OsO_4 at −85 °C for 52 h. Samples were then progressively warmed from −85 °C to 20 °C over 13 h in a Leica automatic freeze substitution unit prior to being washed in acetone and infiltrated and embedded in Araldite resin. Cryo-prepared sample blocks were polymerized underwater using a Pelco Biowave® microwave at 650 W for 90 min. Conventional, microwave-assisted and cryo-prepared sections were imaged unstained on a JEOL 2100 TEM at 120 kV, using a Gatan Orius SC1000 digital camera.

RESULTS AND DISCUSSION

Preservation of adult radulae

While glutaraldehyde fixation of radula epithelium was satisfactory at the LM level when conventional bench-top methods were used, microwave-assisted protocols dramatically reduced sample processing times from six days to one hour (Table 1) and resulted in superior ultrastructural preservation at the TEM level. In addition, microwave-assisted protocols increased fixative penetration into the tooth, improving, for example, tissue preservation within the stylus canal (Fig. 2), which fixed poorly by conventional methods.

The endothelial radula sheath layer, recognizable by the presence of ciliated endothelial cells distributed over the entire sheath's surface, remained intact when processed by microwave methods (Fig. 1), in contrast to conventional methods where it was often disrupted. It is likely that the shorter sample processing and handling times with microwave fixation reduce the likelihood of damage to this delicate membrane. Both techniques preserved prominent vesicles that line the anterior and posterior surfaces of the tooth cusps (Fig. 3). We have found that these vesicles can be either abundant or virtually absent at the same stage of tooth development in different animals. It is currently not known whether these vesicles are natural features of the adult epithelium or artifacts resulting from the fixation process.

Conventional fixation often resulted in the separation of the superior epithelium from the hard tooth cusps, while in microwave-fixed material this artifact was rarely observed (Fig. 3). Despite a number of attempts to improve resin penetration into the base material using both conventional and microwave-assisted methods, including lower resin concentrations and increased infiltration times, the fibrous appearance of the major lateral tooth cusps and stylus persisted and was indeed far more noticeable in microwave-prepared specimens (Fig. 3). This is indicative of poor infiltration by the epoxy resin and is a common problem encountered in mollusc species by many researchers (Nesson and Lowenstam 1985, Mackenstedt and Markel 1987).

Microwave-assisted sample processing resulted in far better preservation of tissue ultrastructure compared to conventional methods. TEM of both conventional and microwave-prepared samples revealed the typical arrangement of organelles within the superior epithelium near the cusp surface that are common to chitons, including microvilli, mitochondria, rough and smooth endoplasmic reticulum, and abundant, electron-dense ferritin siderosomes (Fig. 4). However, at higher magnifications it could be clearly seen

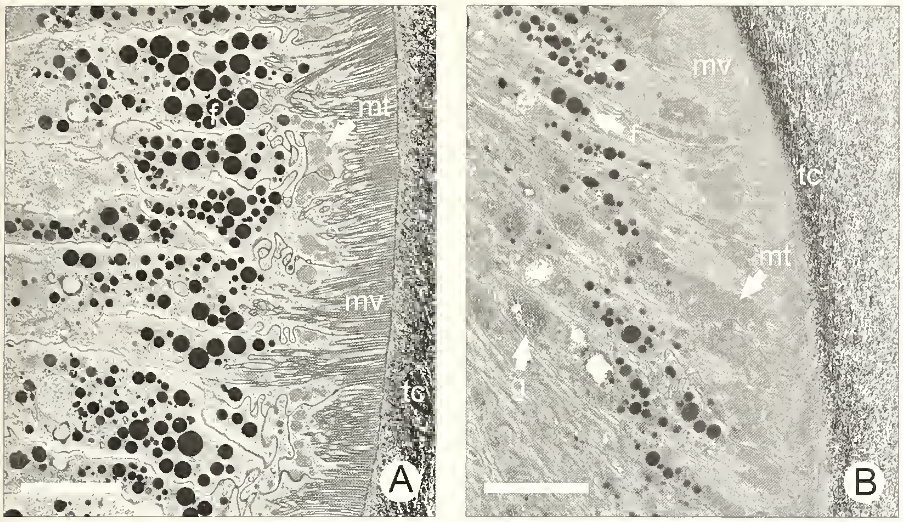

Figure 4. Transmission electron micrographs of the radula superior epithelium abutting the tooth cusp (tc) of (A) conventionally and (B) microwave-processed radulae from adult *Acanthopleura hirtosa* at tooth rows 14 and 13, respectively. f, ferritin siderosome; g, granules; mt, mitochondria; mv, microvilli. Scale bars = 5 μm.

that samples fixed using the microwave contained numerous granules approx. 60 nm in diameter. These granules, which are likely to be either aggregations of ribosomal material or glycogen, appeared throughout the cytoplasm, particularly near the apical poles of the superior epithelium (Fig. 5). These fine structures are either absent or not well preserved in conventional preparations and have not been reported by previous authors (Nesson and Lowenstam 1985, Kim *et al.* 1989). It is likely that the retention of these structures in microwave-prepared samples is a result of the dramatic reduction in processing time of samples, thereby reducing chemical exposure and the chance of extracting soluble components of the tissue.

Preservation of juvenile radulae

The type and arrangement of organelles within the apical epithelium of juvenile *Acanthopleura hirtosa* radulae follows the same characteristic configuration as that described for adult tissue. The preservation quality of juvenile radula epithelium, when using conventional and microwave-assisted methods, was also very similar to that observed in adults (Figs. 6A-B). While the reduction in sample size may improve fixative penetration in juvenile radulae, the comparable fixation quality between adult and juvenile tissue indicates that size is not a limiting factor. In support of this, the ~60 nm granules were absent in conventionally fixed juvenile tissue but were retained in adult and juvenile tissue when processed by the microwave protocol (Figs. 5B, 6B).

With the exception of slight separation of epithelial tissue from the tooth cusps and bases, high-pressure freezing (HPF) resulted in unsurpassed preservation of juvenile *Acanthopleura hirtosa* radula tissue (Fig. 6C). Nuclei (with well defined chromatin adjacent to the nuclear membrane), mitochondria (with well preserved cristae), rough endoplasmic reticulum, and Golgi apparatus were clearly represented in the cytoplasm, together with microvilli and ferritin siderosomes (Fig. 6C). The ~60 nm granules observed in microwave-assisted preparations were also present in HPF samples (data not shown). The large vesicles surrounding the tooth cusps in adult epithelium were also observed in juvenile tissue prepared using conventional and microwave protocols but were absent in HPF material (data not shown). While variations in

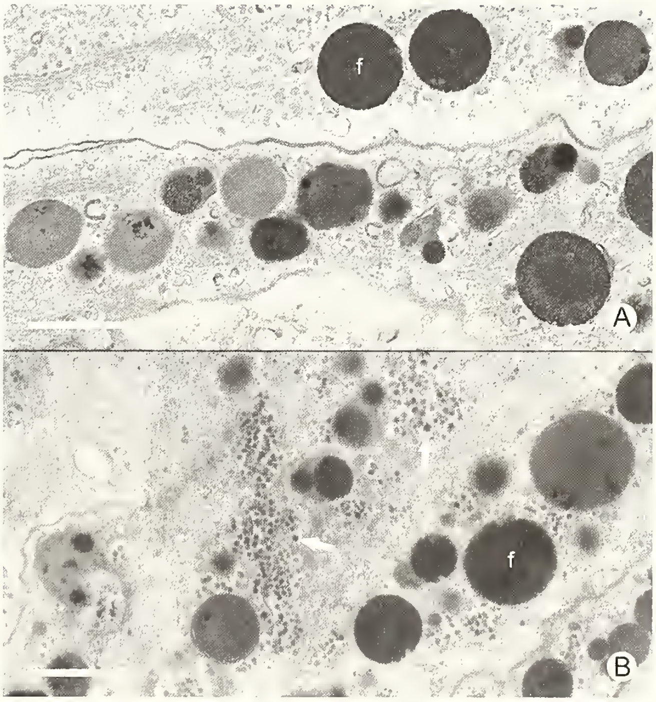

Figure 5. Transmission electron micrograph of (A) conventional and (B) microwave-prepared superior epithelium from adult *Acanthopleura hirtosa* showing differences in the preservation of fine structure. Arrows denote aggregations of ribosomal- or glycogen-like structures within the cytoplasm. f, ferritin siderosome. Scale bars = 1 μm.

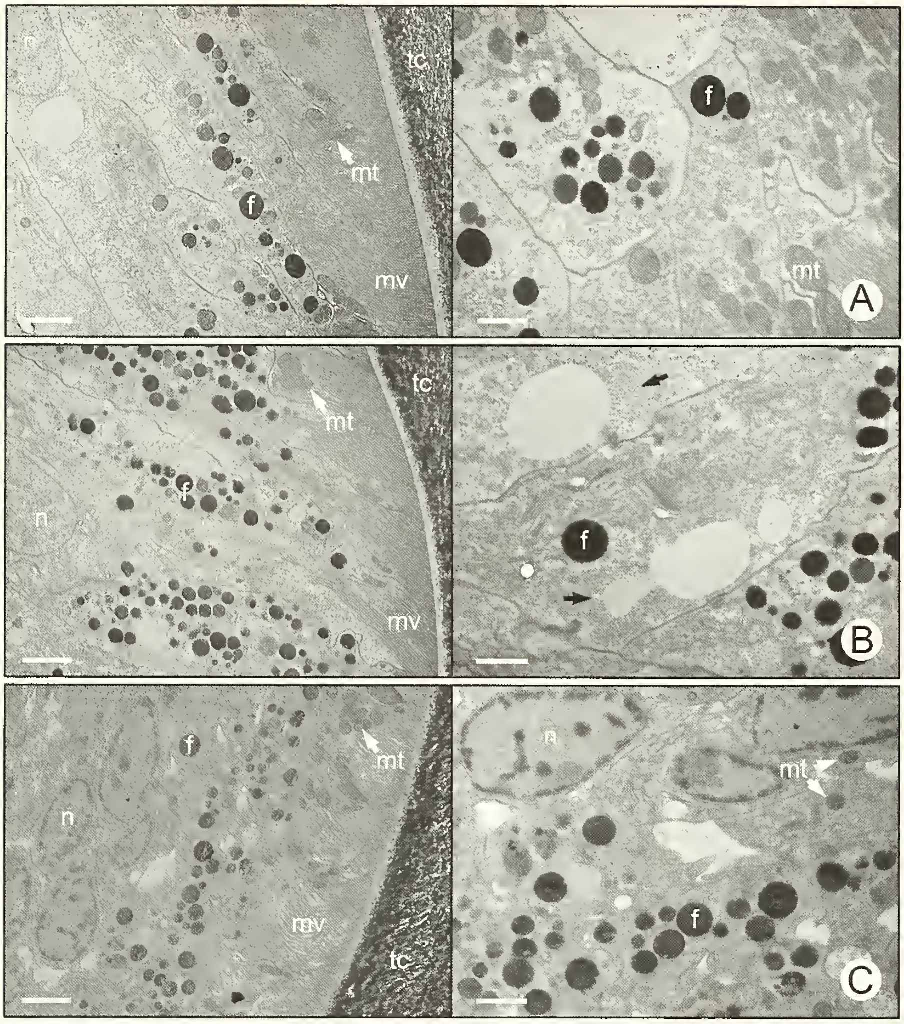

Figure 6. Transmission electron micrographs comparing the preservation of cell ultrastructure in radula epithelium from juvenile specimens of the chiton *Acanthopleura hirtosa* processed using (A) conventional, (B) microwave-assisted, and (C) high-pressure freezing protocols. Black arrows denote aggregations of ribosomes/glycogen. f, ferritin siderosome; mv, microvilli; mt, mitochondria; n, nucleus; tc, tooth cusp. Note: all images were taken from the first tooth row after the onset of mineralization in the cusps. Scale bars = 2 µm (images on left) and 1 µm (images on right).

ultrastructure may arise from differences in the functional state of the cells at the time of fixation (Hayat 2000), it is more likely that these vesicles are artifacts resulting from glutaraldehyde fixation (Bowers and Maser 1988).

Despite a slight improvement in resin infiltration using the conventional method, microwave-assisted processing of radula superior epithelium is preferred due to the improved quality of tissue preservation and the vastly reduced time for sample processing. While cryo-preservation using HPF results in excellent ultrastructural preservation, it is limited with respect to sample size. As such, only the radulae of juveniles or very small mollusc species can be prepared using this method. In addition, the relative portability and affordability of microwave technology compared to HPF makes it a more realistic option for many laboratories. While each of these new techniques has proven to be suitable for fixation of tissue at the LM level, the improved retention of ultrastructural information gained by using microwave and HPF methods highlights the need for a re-evaluation of fine cell structure in molluscan radulae.

While the ultrastructure of the radula epithelium of chitons and limpets has been well documented (Nesson and Lowenstam 1985, Mann *et al.* 1986, Kim *et al.* 1989, Rinkevich 1993), no studies have been conducted on the development of this tissue as teeth progress from an unmineralized to a mineralized state, information that is crucial for resolving the cellular basis of biomineralization. In addition, cryo-techniques have recently been used in conjunction with chemical fixation to characterize the organic matrix in limpets, by dramatically reducing artifacts resulting from staining, dehydration, and embedding (Sone *et al.* 2007). The high-pressure freezing method outlined in the current study therefore provides a valuable first step in preserving the organic matrix for subsequent cryo-sectioning in a frozen hydrated state. The methods of sample preparation presented here not only will benefit future investigations of the superior epithelium and organic matrix of chitons and limpets but also will be of use for taxonomic and morphological studies of molluscan radulae in general.

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Gross anatomy and positional homology of gills, gonopores, and nephridiopores in "basal" living chitons (Polyplacophora: Lepidopleurina)*

Julia D. Sigwart

National Museum of Ireland, Natural History Division, Merrion Street, Dublin 2, Ireland and Queen's University Belfast, School of Biological Sciences, University Road, Belfast, BT7 1NN, Northern Ireland, UK, julia.sigwart@ucd.ie

Abstract: Traditional shell characters are insufficient to differentiate taxa within the polyplacophoran order Lepidopleurida. Additional morphological character sets from soft anatomy (*e.g.*, gamete morphology, gill arrangement, and locations of gonopores and nephidiopores) have previously been described from only a small number of taxa. This study reports for the first time, positions of the gonopores and nephridiopores for 17 species in the Lepidopleurina. The position of both types of pores on the longitudinal body axis varies within a generalized range of the posterior third of the body; however, the separation between the pores as a proportion of the specimen's foot length varies from 3.7% to 17% in different species. Positions of pores relative to the serial gills are also variable within species, and future studies may require a new descriptive basis in order to resolve positional homology. The order Lepidopleurida occupies a critical position with respect to understanding larger-scale patterns in polyplacophoran (and molluscan) evolution.

Key words: *Leptochiton*, Leptochitonidae, descriptive morphology, molluscan evolution

Recent polyplacophorans are divided into two orders: the large, diverse order Chitonida, and the putatively basal Lepidopleurida. All living taxa in this order are grouped into five families within the suborder Lepidopleurina (Sirenko 2006). The group contains around 120 living species in nine genera, although more than eighty of these taxa are traditionally classified in the single genus *Leptochiton* Gray, 1847. All species in Lepidopleurina are characteristically small, without shell insertion plates, rarely with complex shell sculpture or girdle elements, and often found in deep sea habitats including hydrothermal vents (Saito and Okutani 1990), sunken-wood deposits (Sirenko 1988, 1997), and cold-seeps (Kiel and Little 2006). The predominant characteristics of this group could be that they live in the most inaccessible habitats frequented by chitons and are the most difficult to examine once captured.

Detailed understanding of these chitons has progressed slowly; the animals are fundamentally difficult to identify because of their small, plain appearance, and inconsistent nomenclature remains a major source of confusion. For clarity, members of the suborder Lepidopleurina are collectively referred to as "lepidopleurans". The two genus names "*Lepidopleurus*" and "*Leptochiton*" were historically often used interchangeably. There remains persistent confusion over the family name Leptochitonidae Dall, 1889 and its proposed replacement Lepidopleuridae Pilsbry, 1892 (Dall 1889, Pilsbry 1892, Dell'Angelo and Palazzi 1991). Despite the nomenclatural irregularity with the corresponding subordinal and ordinal epithets, the family name Leptochitonidae has clear priority and is the taxonomically correct name for the group.

Although classical descriptions rely on shell characters (or lack thereof), lepidopleurans are also universally allied by their posteriorly arranged gills and apparently simple gametes. Cladistic studies of Polyplacophora have repeatedly recovered the major subordinal groups, with Lepidopleurina as the sister group to other living chitons (Buckland-Nicks 1995, Okusu *et al.* 2003). These studies have also shown that gamete morphology corresponds strongly to these major taxonomic partitions. This corroborates Sirenko's (1993) work correlating gamete structures to gill arrangements for more than 100 species; although Sirenko (1993) reported the position of nephridiopores and gonopores for 130 species in the Chitonida, he included no lepidopleurans.

To date, the gamete morphology for seven species of lepidopleuran chiton have been published: *Leptochiton asellus* (Gmelin, 1791), *Leptochiton rugatus* (Capenter MS, Pilsbry, 1892), *Leptochiton assimilis* (Theile, 1909), *Deshayesiella curvata* (Capenter MS, Pilsbry, 1892), *Hanleya hanleyi* (Bean in Thorpe, 1844), *Hanleyella asiatica* Sirenko, 1973 (Sirenko 1993, Hodgson *et al.* 1988, Buckland-Nicks 2006). Of those, most have smooth egg hulls, but *Hanleyella asiatica* has long hair-like chorion processes on the egg hull, similar to other (unrelated) brooding species. However, work on other brooding species with reduced chorion processes indicates that egg hulls show little homoplasy (Sirenko 1993, Eernisse and Reynolds 1994). The lepidopleuran sperm type is also considered plesiomorphic as it lacks the relatively complex acrosome processes characteristic of all described sperm from non-lepidopleuran chitons (Buckland-Nicks 2006).

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Gamete characteristics are of great interest, but examination is limited in a practical way by fixation and specimen availability. The gross morphology of the exterior position of the gonopore, which can be examined on preserved specimens, may also be related to fertilization and reproductive biology. The gonopore and nephridiopore in all chitons are located on the roof of the pallial cavity between the gills (Fig. 1).

More than 50% of lepidopleuran taxa are found at a minimum depth of at least 100 m, and many recently described species in the Leptochitonidae have a maximum adult length of 5 mm or less. However, it is still not understood how many morphological features may respond to environmental conditions within the lifespan of the individual animal.

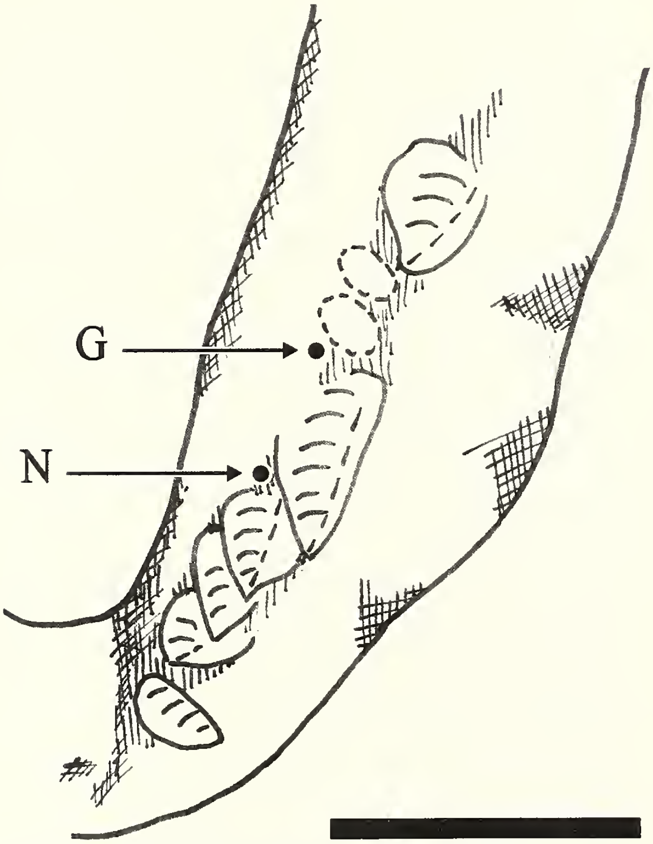

Figure 1. Camera lucida drawing of *Leptochiton norfolcensis* (Hedley and Hull, 1912); scale bar = 5 mm. Note nephridiopore (posterior) between gill 4 and 5, and gonopore between gill 5 and 6 (gill 6 and 7 removed, indicated by dashed outline).

This paper presents a series of preliminary descriptions on the position, number, and distribution of gills, gonopores, and nephridiopores across a sample of basal living chitons. Little previous work of this type has been conducted, especially in these very small animals; it is hoped that a survey of this kind will initiate additional morphological (especially microanatomical) and phylogenetic studies within Lepidopleurina. There is little doubt that this clade of chitons will prove of critical importance to our understanding of polyplacophoran evolution.

MATERIALS AND METHODS

This study included examination of the alcohol-preserved material in the former collection of P. Kaas (1915-1996) held in Naturalis, Leiden, The Netherlands (RMNH) and that of R. A. Van Belle (1920-2005) in the Royal Belgian Institute of Natural Sciences, Brussels, Belgium (RBINS). These two collections make up a substantial proportion of the fluid-preserved polyplacophoran material in their respective institutions. The majority of specimens were historically fixed in formalin before being transferred to methylated ethyl alcohol (70%); some are presumed to have been additionally preserved with glycerin. Both collections include material that was collected by Kaas and Van Belle as well as other colleagues who contributed material to their private collections. The specimens vary in collecting age from the 1970s to the 1990s. As the majority of specimens were collected by persons knowledgeable about chitons and were flattened at preservation, they are in excellent condition for morphological examination.

All specimens that were preserved in a flattened posture were examined under a dissecting microscope (up to 100× power) for observing the number of gills per side. The location of the gonopores and nephridiopores relative to gills was determined without dissection, usually by using 000 gauge insect pins as a probe to separate gills for inspection of the pallial cavity roof. Use of flexible insect forceps allowed the specimen to be held in place without damage and while immersed in fluid. In some cases for specimens in RBINS, a small strip of tissue was removed from the lateral margin of the foot at the posterior end, to facilitate viewing the gill row. In other cases for specimens in RBINS and RMNH, individual gills were removed ("pinched" off) to facilitate viewing the pallial cavity roof. Multiple specimens in a species were examined where suitable material was available.

Position of nephridiopores and gonopores was recorded relative to the posterior end of the gill row. Gills were numbered from the one closest to the anus and counted in series, following Sirenko (1993). Positions of the pores were recorded as between the two closest gills, or in some cases, one gill when the pore was directly in front of the base of a single gill.

Measurements to the nearest 0.1 mm were taken with calipers for total body length, foot length, and length of the gill row. As the number of gills may vary between left and right sides of individual specimens, all pore placements were confirmed on both sides; gill counts and measurements were taken uniformly from the left side of the animal. These measurements were omitted where specimens were damaged or too curled to allow accurate measure. Rapid degradation of the external gill tissue after death means that only the best-preserved specimens were suitable for determination of the location of the gonopores and nephridiopores by this technique. The separation between gonopore and nephridiopore was estimated based on the number of gills separating pore locations and the average inter-gill distance (length of row divided by number of gills per side). This is possible with some degree of accuracy, as pores are consistently placed in the middle portion of the gill row, where each individual gill is of "average" width. All measurements reported were taken from a mean of three successive caliper measures.

Table 1. Location of nephridiopore relative to closest gills (counted from posterior) and number of gills per side in specimen.

Species	Nephridiopore	Gonopore	Gills
Leptochiton algesirensis (Capellini, 1859)	5-6	8-9	15
Leptochiton algesirensis	5-6	9-10	16
Leptochiton algesirensis	6-7	9-10	16
Leptochiton algesirensis	7-8	10-11	16
Leptochiton algesirensis	7-8	11-12	16
Leptochiton asellus (Gmelin, 1791)	5-6	7-8	10
Leptochiton asellus	5-6	7-8	10
Leptochiton asellus	5-6	7-8	10
Leptochiton badius (Hedley and Hull, 1908)	4-5	5-6	8
Leptochiton belknapi Dall, 1878	5-6	7-8	14
Leptochiton cancellatus (Sowerby, 1840)	5-5	6-7	8
Leptochiton cancellatus	5-6	6-7	8
Leptochiton cancellatus	5-6	6-7	8
Leptochiton cancellatus	5-6	7-7	8
Leptochiton cancellatus	5-6	6-7	8
Leptochiton foresti (Leloup, 1981)	7-8	8-9	10
Leptochiton kurnilatus Kaas, 1985	3-4		6
Leptochiton micropustulosus Kaas, 1994	9	10-11	14
Leptochiton micropustulosus	9	10-11	14
Leptochiton micropustulosus	9-10	12-13	14
Leptochiton norfolcensis (Hedley and Hull, 1912)	4-5	5-6	8
Leptochiton norfolcensis	4-5	5-6	8
Leptochiton rugatus (Carpenter in Pilsbry, 1892)	5-6	7-7	10
Leptochiton rugatus	5-6	6-7	10
Leptochiton sarsi Kaas, 1981	5-6	7-8	9
Lepidopleurus cajetanus (Poli, 1791)	7	9-9	14
Lepidopleurus cajetanus	7-8	8-9	14
Parachiton eugenei (Kaas and Van Belle, 1985)	7-8	10	16
Parachiton hylkiae Strack, 1993	7-8	11-12	16
Parachiton hylkiae	8-9	10-11	16
Parachiton politus Saito, 1996	10-11	12-13	16
Nierstraszella lineata (Nierstrasz, 1905)	9-10	10-11	14
Nierstraszella lineata	9-10	10-11	14
Nierstraszella lineata	8-9	13-14	16
Nierstraszella lineata	8-9	13-14	16
Nierstraszella philippina (Leloup, 1981)	5-6	7-8	12

RESULTS

A total of 49 species from eight lepidopleuran genera were examined for placement of gonopore and nepridiopore; however, pore locations relative to the gills were successfully determined in only 17 species (Table 1). The specimens ranged from 4.7 mm to 20.6 mm in body length. Specimens had between 4 to 21 gills per side, and the gill row ranged in length from 24% to 68% of foot length (but the median value of 40% of foot length was characteristic of the suborder).

Taxa for which pore locations were successfully determined include the genera *Leptochiton* (11 species), *Lepidopleurus* (one species), *Parachiton* Thiele, 1909 (three species), and *Nierstraszella* Sirenko, 1992 (two species), represented by a total of 36 specimens. These specimens ranged in size from 5.1 mm to 15.1 mm (Table 2) and had between 6 to 16 gills per side (Table 1). The general range of placement of the gonopore and nephridiopore as a pair was variable throughout all taxa (Fig. 2). Although the two orifices have no physiological relationship, their placement and relationship with

Table 2. Size of chiton specimens; total body length measured on dorsal side in flattened position; foot length measured ventrally from mouth to anus; gill row measured on right-hand side from anus or posterior-most gill (ctenidium) to anterior-most gill.

Species	Body length (mm)	Foog length (mm)	Gill row length (nm)	Interpore distance (mm)
Leptochiton algesirensis (Capellini, 1859)	8	5.1	2.1	0.39
Leptochiton algesirensis	13.5	10	4.6	0.86
Leptochiton asellus (Gmelin, 1791)	7.8	5	1.9	0.38
Leptochiton badius (Hedley and Hull, 1908)	6	4.2	1.3	0.16
Leptochiton cancellatus (Sowerby, 1840)	5.9	3.7	1.1	0.14
Leptochiton cancellatus	7.1	4.7	1.5	0.19
Leptochiton foresti (Leloup, 1981)	6.5	4	2	0.20
Leptochiton micropustulosus Kaas, 1994	12.5	8.8	3.5	0.50
Leptochiton micropustulosus	14.6	9.2	4	0.57
Leptochiton micropustulosus	15.1	10.2	3.6	0.77
Leptochiton norfolcensis (Hedley and Hull, 1912)	5.1	3.1	1.1	0.14
Leptochiton rugatus (Carpenter in Pilsbry, 1892)	7.9	5.6	2	0.30
Lepidopleurus cajetanus (Poli, 1791)	11.5	8.5	3	0.43
Parachiton eugenei (Kaas and Van Belle, 1985)	6.8	5	2	0.31
Parachiton hylkiae Strack, 1993	12.5	7.3	5.0	1.25
Parachiton hylkiae	9.8	6.3	3.7	0.46
Parachiton politus Saito, 1996	10.4	7	3.7	0.46
Nierstraszella lineata (Nierstrasz, 1905)	13.9	9.1	5	1.56
Nierstraszella lineata	13.9	9.1	5	1.56

the respiratory cavity allow us to consider them collectively for convenience here.

The placement of nephridiopores and gonopores relative to the posterior-most gill was variable among species and, in some cases, among presumed conspecifics. The majority of species in all genera (11 of 17) had a nephridiopore at the sixth or seventh gill (counted from posterior of the gill row). Other species with more posterior pores (relative to the gill row) had fewer gills–six or eight per side. However, species with more anterior pores were not correlated to higher gill counts. Although most species retained consistent pore positions in multiple specimens, some (*Leptochiton algesirensis*, *Nierstraszella lineata*) varied between individuals.

The separation between nephridiopore and gonopore was also highly variable. As an estimated proportion of the length of the foot, the gap varied between 3.7% to 17% with the widest separation in *Nierstraszella lineata* and one specimen of *Parachiton hylkiae* (Table 2). This was radically different from the other specimen of *Parachiton hylkiae* for which data was available (with an interpore separation of only 7.3% of the foot length) and *Parachiton politus* (6.6%). The relationships of these proportions were similar whether considered relative to the gill row or total body length.

All species examined had gills of the adanal condition (*sensu* Sirenko 1993), having multiple gill pairs posterior of the nephridiopore. Some *Leptochiton* species would be considered "abanal" (*sensu* Plate 1899) in that all gills were of equal length; however, these taxa were adanal (*sensu* Sirenko) as are all lepidopleurans.

DISCUSSION

The locations of gonopores and nephridiopores were considerably variable with respect to gill placements in lepidopleurans, which concurs with variability that has been reported for other chitons (Sirenko 1993). The position of the gonopore in the taxa examined was not fixed, neither positionally relative to the gill row nor in distance to the nephridiopore. This variability must be constrained by the internal organ systems but external variability is indicative of internal variability. The change in pore placement and the effects on respiration, reproduction, and excretion may mark physiological differences that impact the daily life of the animals. Variations in pore placement do not appear to be consistent with currently recognized genera, as they differ within as well as among genera.

Sirenko (1997: 13) reported pore locations for nine lepidopleuran taxa (but did not comment on these results in the discussion of his paper). None of these taxa were duplicated by this survey. However, Sirenko (1997) included two species of *Parachiton*, both of which had more than 20 gill pairs and found nephridiopore (11-12) and gonopore (12-13)

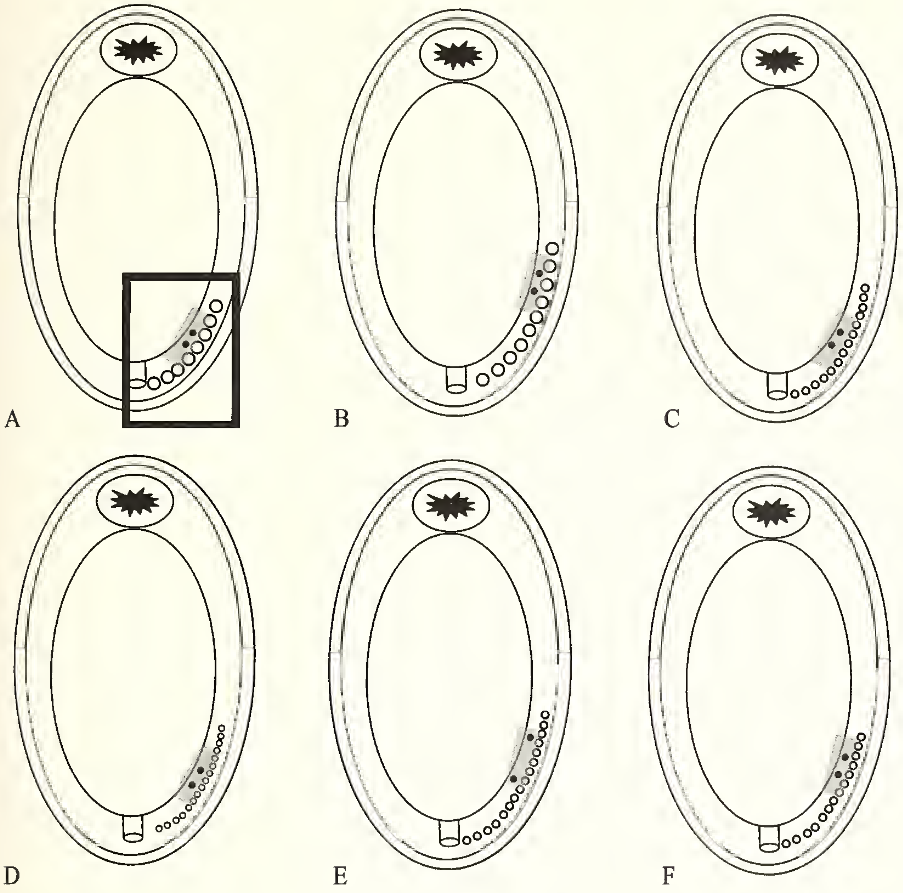

Figure 2. Schematic drawing of chiton ventral surfaces and the placements of gonopores (anterior) and nephridiopores (posterior). Drawings are stylized to emphasize positions of gills relative to gills only; the position of the nephridiopore is consistently underneath valve VII of the animal. A, *Leptochiton norfolcensis*, inset area illustrated in Fig. 1; B, *Leptochiton foresti* (Leloup, 1981); C, *Lepidopleurus cajetanus* (Poli, 1791); D, *Parachiton eugenei* (Kaas and Van Belle, 1985); E, *Nierstraszella lineata* (Nierstrasz, 1905); F, *N. lineata*.

placements that differ from the species examined in this study.

Within this morphological survey, species showed different patterns of variability. For instance, most specimens examined of *Leptochiton algesirensis* had the same number of gills per side, yet there are four different pore arrangements associated with 16 gills. This contrasts with the other most sampled species, *Leptochiton asellus*, which had extremely consistent pore positions across all specimens. In other cases there may be morphologies associated with growth patterns (or potentially cryptic species): in *Nierstraszella lineata* there is one positional morphology associated with 14 gills and another quite distinct pore arrangement in specimens with 16 gills.

The polyplacophoran nephridiopore is consistently located underneath valve VII, and the gonopore is anterior to the nephridiopore. Non-lepidopleurans have a substantial gap between the anus and the gills, and the nephridiopore is at the posterior end of the gill row (between 1 and 2; Sirenko 1993). In lepidopleurans, however, the nephridiopore may be central within or even anterior to the gill row. This does not necessarily represent anatomical change in the pore, but rather shift in the gill arrangement morphology. Although constrained to the region under valve VII, the nephridiopore clearly varies in position relative to the body as well, as illustrated by considering the placement relative to the proportional measurement of the foot length. This should not be a surprise; for example, *Parachiton* has a characteristic enlarged tail valve, which takes up a larger proportion of the animal's foot length than in other lepidopleurans. The nephridiopore in *Parachiton* is underneath valve VII, but the nephridiopore is necessarily more anterior relative to the foot (and potentially other organ systems) than in other genera.

The variability of gill arrangements in chitons was first described by Plate (1899) by differentiating extent of the gill row (all lepidopleurans are approximately "merobranchial" with posteriorly arranged gills), presence of small gills posterior of the major gill row, and the presence of a gap between the posterior-most gill and the anus. Ontogenetically, the adanal arrangement means that new gills are added to both ends of the gill row during growth, whereas abanal gill rows grow in an anterior direction only (with the oldest and usually longest gills at the posterior). Contemporary 19th century work proposed that the "adanal" state characterized by extra small gills at the posterior end of the row, typical of lepidopleurans, represents the plesiomorphic condition (Pelseneer 1899).

Sirenko (1993) improved Plate's (1899) classical definitions of "abanal" and "adanal" gill arrangements by reinterpreting them with reference to the nephridiopore. This usage effectively segregates the lepidopleuran taxa as an adanal clade. In the majority of lepidopleuran species, the posterior-most gills are small and sometimes bud-like processes near or attached to the anus. These minute gills are visible on individual animals of adult size. Ontogenetically, in some species, these may grow to determinant size, but they may also be influenced by environmental plasticity, growing to suit local respiratory requirements. Kaas and Van Belle (1985) noted that counts of gills were variable in adult specimens of some species, and that counts of gills on right and left sides may vary within a single individual. Observationally, it appears that comparisons within taxa are not negatively affected as multiple individuals of the taxa included in this survey and others do have a consistent number of gills although the marginal gills may be very different in size. These structures illustrate that gills within a gill row on an individual chiton are not equal and interchangeable units.

Sirenko (1993) proposed using the posterior-most gill as a reference point for describing the location of nephridiopores and gonopores, as followed in this survey. In abanal chitons, the gill row grows in the anterior direction, making posterior-based counting a consistent method regardless of the animal's growth state. As adanal gill arrangement is presumed indicative of bi-directional growth of the gill row, using the posterior-most gill as a reference point may not be sufficient in these taxa. Examination of a single specimen may not give a positional result that can be validated in an older individual. The question of ontogeny is not fully resolved; gill row growth may be bi-directional but examination of juvenile specimens shows that the primary direction of growth is still anterior. The gill "buds" at the posterior end of the gill row appear early in post-settlement individuals. These buds are interpreted to be new gills that have not achieved full length; however, they appear in many adult sized specimens which brings into question whether some species have determinate growth in gill numbers. In examination of the gill row in a chiton of indeterminate age (as they all are), there is also no way to determine which individual gill of full size marks the "beginning" point of growth.

A number of South Pacific lepidopleurans, primarily those described by Sirenko (2001) and other related taxa, have only four gills. (Interestingly, these species would be considered "abanal" *sensu* Plate because all four gills are equal in length, but there is no question that the species are lepidopleurans). Because the entirety of the gill row is located underneath the tail valve, both the nephridiopore and gonopore are probably located on the naked pallial roof. This is a clear limitation on the present methods for describing the anatomy of nephridiopores and gonopores externally: there is no practical way to describe their placement or compare placements among these species.

Correlation of gonopores and nephridiopores, as well as internal anatomical structures, to shell morphology requires further descriptive work. Measurement of position in this case and in other reports is recorded relative to gills, but it should additionally be measured relative to dorsal valve and sutural positions. Some of the variability observed in this morphological survey is probably an artifact of bi-directional gill row ontogeny. However, there is clearly a high level of plasticity that cannot be completely accounted for (particularly in the well-sampled case of *Leptochiton algesirensis*) without acknowledging that the pores, and therefore internal organ anatomy, are variable within and between taxa to a higher degree than previously recognized.

In this group, as in many groups of invertebrates where species identification is challenging, new characters that help differentiate taxa are always welcome. However, the problems illustrated here demonstrate that either pore placement relative to gill elements is not positionally homologous or, alternatively, if pore placements are apomorphic then current genera are not monophyletic assemblages. Variability in this and other soft anatomical features in lepidopleurans may mask cryptic species not identifiable by traditional shell and girdle characters.

The inconsistent morphology within recognized genera in the preliminary results reported in this paper suggest that these structures may not be positionally homologous. Potential plasticity of elements in the gill row in lepidopleuran chitons is not sufficiently known and means that the positions of pores with respect to gills may be homoplasious for phylogenetic inference. It will be interesting to compare the results presented here with future hypotheses about internal relationships within the lepidopleuran clade.

Although these chitons are superficially similar, this ancient lineage of molluscs shows a great deal of adaptation to different marine environments. This is reflected in morphological differentiation, although not in the traditional shell and girdle characters used to describe polyplacophoran species. The issues raised in this survey indicate that although the nephridiopores and gonopores of lepidopleuran chitons are superficially similar in structure, our current means of identifying and describing their placement is insufficient to consider them to be positionally homologous across taxa for the purpose of systematic inference. The patterns of variance are too clouded by our poor understanding of morphology to shed more light on internal relationships within this group; however, the morphological variation observed here is undoubtedly linked to the high level of evolutionary variance in this diverse group of chitons. Lepidopleurans lack the dramatic colors and patterns of their sister-group the Chitonida, but nonetheless represent a diverse group of marine organisms.

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Aesthete canal morphology in the Mopaliidae (Polyplacophora)*

Michael J. Vendrasco[1], Christine Z. Fernandez[2], Douglas J. Eernisse[3], and Bruce Runnegar[4]**

[1] Institute for Crustal Studies, 1140 Girvetz Hall, University of California, Santa Barbara, California 93106-1100, U.S.A., mvendrasco@fullerton.edu

[2] 14601 Madris Ave., Norwalk, California 90650, U.S.A.

[3] Department of Biological Science (MH-282), California State University, Fullerton, P.O. Box 6850, Fullerton, California 92834-6850, U.S.A.

[4] Department of Earth and Space Sciences, Institute of Geophysics and Planetary Physics and Molecular Biology Institute, 595 Young Drive East, University of California, Los Angeles, California 90095, U.S.A.

Abstract: The aesthete canals of fourteen chiton species were cast with epoxy, allowing detailed examination and comparison of the entire canal system that infiltrates their valves (shell plates). Some species in this study have been classified without question in the family Mopaliidae (*Mopalia ciliata* (Sowerby, 1840), *Mopalia lignosa* (Gould, 1846), *Mopalia spectabilis* Cowan and Cowan, 1977, *Mopalia swanii* Carpenter, 1864, *Katharina tunicata* (Wood, 1815)), while other species have been placed in that family by some workers but not others (*Dendrochiton flectens* (Carpenter, 1864), *Dendrochiton lirulatus* (Berry, 1963), *Tonicella insignis* (Reeve, 1847), *Tonicella lineata* (Wood, 1815), *Tonicella lokii* Clark, 1999, *Tonicella marmorea* (Fabricius, 1780), *Nuttallochiton mirandus* (Thiele, 1906), *Plaxiphora aurata* (Spalowski, 1795)), and one has never been placed in the Mopaliidae (*Tonicia chilensis* (Frembly, 1827)). The results provide additional evidence that there is high diversity in aesthete canal morphology but also some striking resemblances interpreted here as homologies, reaffirming that aesthete canal characters have considerable potential for phylogenetic analyses and for supporting classification ranks ranging from suborder to species. In this case, the results are broadly consistent with traditional classifications of mopaliids, but *Tonicella* and *Dendrochiton* (taxa not always thought not to be mopaliids) share many aesthete canal synapomorphies with undisputed mopaliids, whereas *Plaxiphora* (typically thought to be a mopaliid) has an aesthete canal system more similar to non-mopaliid members of the Acanthochitonina. These differences are in line with results of recent phylogenetic analyses of the Mopaliidae.

Key words: Chiton, *Mopalia*, valve, tegmentum, esthete

The hard layers of chiton valves consist of the uppermost tegmentum, the articulamentum whose projections form the sutural laminae and insertion plates, and the underlying hypostracum. The tegmentum, which is the visible layer of the chiton shell in life, is infiltrated with a complex, tissue-filled canal system that opens at the dorsal valve surface as sensory or secretory organs known as aesthetes (also *esthetes*) (Marshall 1869). The pores on the dorsal surface are entrances of tiny canals that often pass into bulb-shaped (aesthete) chambers that then connect to larger horizontal canals and eventually exit at the valve's anterior or lateral margin, or in some regions of the ventral valve surface (now known to correspond to the nervous innervation of the aesthetes). Knorre (1925) made detailed schematic drawings of the entire canal system in *Lepidochitona cinerea* (Linnaeus, 1767) (as *Trachydermon cinereus*) that revealed this configuration of canals. Prior to Knorre's (1925) work, Moseley (1885) noticed two size classes of aesthete pores (termed micropores and megalopores) on the dorsal valve surface and coined the term megalaesthete for the organic tissues within the often bulbous chambers near the valve's dorsal surface, and micraesthete for the tissues in the smaller canals that connect from the bulb of the megalaesthete to the valve surface. The organs that occupy the upper portion of the chiton tegmentum include the aesthetes (Blumrich 1891) and in some cases also the extrapigmental and intrapigmental ocelli (Nowikoff 1907, 1909). Although aesthetes have been found in all modern chitons so far examined, ocelli have so far been found only in some members of the Schizochitonidae (Moseley 1885) and Chitonidae (Boyle 1977).

Although there have been numerous studies of aesthetes in many chiton species (see the review in Reindl *et al.* 1997),

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** Present address: Department of Biological Science (MH-282), California State University, Fullerton, P. O. Box 6850, Fullerton, California 92834-6850, U.S.A.

their function has been debated. Moseley (1885) documented the morphology of ocelli in *Schizochiton incisus* (Sowerby, 1841) (Chitonina: Schizochitonidae). In this species, the ocelli are the largest known for any chiton and are sparsely distributed in relatively huge chambers—presumably enlarged megalaesthete cavities. Boyle (1969a, 1969b) confirmed the presence of photoreceptors in ocelli in *Onithochiton neglectus* Rochebrune, 1861. A photosensory role for aesthetes had initially been proposed by Blumrich (1891) and observations that certain chiton species are either positively or negatively phototactic (*e.g.*, Crozier 1920, Omelich 1967, Boyle 1972, Fischer 1988, Currie 1989) have led many to view photoreception as the primary role of aesthetes. Indeed, Crozier and Arey (1918) observed that a crawling *Chiton tuberculatus* Linnaeus, 1758 (which lacks large conspicuous ocelli) immediately stopped crawling, temporarily, in response to a shadow from a fly about 2 m away. However, the function of most of the aesthetes, besides those with ocelli, has been disputed, with suggestions including mechanoreception (Moseley 1885), chemoreception (Fischer 1988, Baxter *et al.* 1990), periostracum replenishment and secretion (Boyle 1974, Baxter *et al.* 1987, 1990), and secretions for protection, prevention of desiccation, or fouling by epibionts (Fischer 1988). An electron microscopy study of aesthete tissues (Omelich 1967), an immunocytochemical study (Reindl *et al.* 1997), and electrical recordings (Omelich 1967, Fischer, pers. comm. in Eernisse and Reynolds 1994) have all shown that aesthetes contain neuronal structures, demonstrating a sensory function in at least some cases. However, it seems plausible that aesthetes could serve many roles, or the functions differ in different lineages, as suggested by Haas and Kriesten (1978), Fischer (1978, 1988), Sturrock and Baxter (1995), and others.

Because features of the aesthete canal system and the nature of aesthete caps vary between chiton taxa (*e.g.*, Boyle 1974, Baxter and Jones 1981, 1984, Sturrock and Baxter 1993, 1995, Reindl *et al.* 1997, Schwabe and Wanninger 2006), they provide a suite of characters that have been included in phylogenetic and taxonomic studies of chitons (*e.g.*, Hull and Risbec 1930-1931, Leloup 1940, 1942, 1948, Bullock 1985, 1988, O'Neill 1985, Watters 1990, Sirenko 1992, 2001, Saito 1996, 2006). Brooker (2004) included extensive data from the distribution, arrangement, density, shape, and size of ocelli; aesthete pore area, shape, densities, and ratios; and size, density, and shape of large pores in the tegmentum eaves in her cladistic analysis of the *Acanthopleura* Guilding, 1829. Moreover, Schwabe and Wanninger (2006) documented variation among chiton genera in the elevation of aesthete pores, pigmentation in the megalaesthetes, and the arrangement of micraesthetes around the megalaesthetes.

Currie (1989), however, cautioned that there can be much variation in size and density of aesthete pores in different areas of one valve. This point was echoed by Brooker (2004), who found this to be true in the *Acanthopleura* and who emphasized the need to compare data from the same valve area when describing differences in aesthete patterns between species.

Recently, using an approach pioneered by Haas and Kriesten (1978; see Eernisse and Reynolds 1994), Fernandez *et al.* (2007) made epoxy casts of the aesthete canal system in twelve chiton species and demonstrated variation among suborders, families, genera, and species. A cladistic analysis revealed congruence between relationships inferred from aesthete canal characters alone and those derived from other aspects of morphology as well as molecules, suggesting that aesthete canal characters are useful in helping to infer relationships between chitons (Fernandez *et al.* 2007).

This study expands previous work by focusing largely on internal relationships within one family of chitons, Mopaliidae Dall, 1889. This family has conventionally included at least *Mopalia* Gray, 1847, *Placiphorella* Carpenter in Dall, 1879, *Amicula* Gray, 1847, *Katharina* Gray, 1847, *Plaxiphora* Gray, 1847, and *Placiphorina* Kaas and Van Belle, 1994 (*e.g.*, Kaas and Van Belle 1994, Sirenko 2006). The placement of *Nuttallochiton* Plate, 1899 and *Dendrochiton* Berry, 1911 has been less consistent. For example, Thiele (1931) placed *Nuttallochiton* in the Lepidochitonidae, whereas Van Belle (1983) and Kaas and Van Belle (1987) placed it in the Chaetopleurinae (Ischnochitonidae) instead. Sirenko (1993, 1997, 2006) later assigned this genus to the Mopaliidae, although recent molecular phylogenetic analyses (Okusu *et al.* 2003, Eernisse, unpubl. data) suggest that *Nuttallochiton* should be excluded from that family. Berry (1911) originally proposed *Dendrochiton* as a member of Mopaliidae because its members possess girdle setae similar to those of *Mopalia*, but others have instead considered it to belong to Lepidochitonidae (*e.g.*, Ferreira 1982, Van Belle 1983). Van Belle (1983) and Kaas and Van Belle (1985) even considered it to be a subgenus of *Lepidochitona* Gray, 1821 within Lepidochitonidae.

Eernisse (unpubl. data) used mitochondrial 16S rDNA data to discover a previously unrecognized association of some conventional mopaliid genera (*Placiphorella*, *Katharina*, *Amicula*, and *Mopalia*) with genera normally placed in other families (*Cryptochiton*, *Tonicella* Carpenter, 1873, and *Dendrochiton*). Furthermore, these analyses revealed that the more southern genera, *Plaxiphora* and *Nuttallochiton*, are only distantly related to Mopaliidae. This family was previously diagnosed by a posterior caudal sinus in the tail valve, which may instead be interpreted as a convergent trait related to size increase and the enhancement of respiratory currents (Eernisse, unpubl. data). In order to provide evidence for or against this proposed rearrangement, we examined aesthete canal morphology in a number of undisputed mopaliid taxa in addition to representatives newly embraced into, or excluded from, the Mopaliidae based on this new classification scheme (Eernisse, unpubl. data).

Thus the goals of this study were to: (1) determine if aesthete canal morphology supports this new taxonomic scheme of the Mopaliidae; (2) determine the degree and nature of variation in aesthete canal systems in a larger set of chitons (see Figs. 1 and 2) to better assess the hypothesis in Fernandez *et al.* (2007) that such characters are useful in chiton phylogeny; and (3) use the new data to refine the previous attempt (in Fernandez *et al.* 2007) to define potential characters and states of the aesthete canal system that may be useful in future phylogenetic analyses of chitons.

MATERIALS AND METHODS

All chitons used in this study were adults and most were collected from the Eastern Pacific (collection data: Appendix 1). Valves from at least two individuals from each species were treated, except for the deep-water *Nuttallochiton mirandus*. Two or three intermediate valves of each individual were embedded and examined. All epoxy casts and voucher valves for each species in this analysis have been deposited at the Santa Barbara Museum of Natural History (SBMNH).

Valves were removed from dried or alcohol-preserved specimens using a scalpel, tweezers, and scraping tools. Boiling the chitons to remove the valves was not done because this can break valves into pieces. The isolated intermediate valves of all species were soaked in household bleach for up to 24 hours and placed in a sonicating bath for 20-30 minutes at room temperature to dislodge remnant organic material and other debris. Valves were dehydrated through an ethanol series and then embedded in epoxy using a method modified after Golubic *et al.* (1970). A low viscosity, medium hardness embedding medium was mixed using the Embed 812 kit from Electron Microscopy Sciences. The Embed 812 kit consisted of Embed 812 embedding resin, Dodecenyl Succinic Anhydride (DDSA), Nadic Methyl Anhydride (NMA), and Benzyldimethylamine (BDMA). They were combined in the following proportions: 44.2% Embed 812, 35.4% DDSA, 17.7% NMA, and 2.7% BDMA. The valves were submerged in resin and placed under a vacuum in a desiccating chamber for 24 hours and then cured in an oven at 60 °C for 24 hours. The cured epoxy blocks were trimmed using a rotary hand tool with a thin-bladed saw. Cuts were made around the edges of the valves, making sure to intersect the valve along much of its margin. The epoxy blocks were placed in 10% HCl for another 24 hours, or until all of the calcium carbonate in the valves dissolved away, then rinsed thoroughly with distilled water, cleaned with bleach, and split apart into a dorsal and ventral cast.

In a few cases (*e.g.*, Fig. 3F), after the vacuum stage but prior to curing, valves were drained of most epoxy, with only a shallow pool extending just above the insertion plates. The surface of these valves, still with a coating of epoxy, was then wiped with a Kimwipe dipped in alcohol. This alternative method was included to better see the meglaesthete bulbs and associated micraesthetes near the dorsal valve surface (it allows the aesthete chambers to be visible from the top, rather than from underneath, where they are largely hidden by underlying canals).

The casts were gold sputtered for 90 seconds and examined using a LEO 1430 Scanning Electron Microscope (SEM) with an accelerating voltage (EHT) of 10-15 kV under high vacuum. Many images were taken using backscatter electron detectors (two to four quadrants) at high or variable pressure. The backscatter detectors (QBSD) produced far less charging than occasionally occurred with the secondary electron detector (SE). The number and exact backscatter detectors varied, though the best contrast was usually achieved with 3 of 4 detector quadrants on. In a few cases, charging still occurred, so variable pressure (30-40 Pa) and the Variable Pressure detector (VPSE) were used.

In many species, mopaliids in particular, the dense carpet of horizontal canals on the dorsal casts prevented a clear view of the overlying (in life; in SEM photographs of the dorsal casts, they underlie the horizontal canals), near-surface canal system. In these cases, a thin-tipped needle was used to pull away some of the horizontal canals and reveal the megalaesthete chambers and micraesthete canals that lay below on the casts. In such cases, re-coating with gold was necessary.

Epoxy casts made prior to this study and described in Fernandez *et al.* (2007) were also used in comparative analyses herein. These specimens are: *Mopalia muscosa* (Gould, 1846) (SBMNH 83143 and 83144), *Mopalia acuta* (Carpenter, 1855) (SBMNH 83160 and 369432), *Cyanoplax* (as *Lepidochitona*) *hartwegii* (Carpenter, 1855) (SBMNH 83146 and 83147), *Nuttallina californica* (Nuttall MS, Reeve, 1847) (SBMNH 83148, 83149, and 83156), *Lepidozona cooperi* (Dall, 1879) (SBMNH 83150 and 83151), *Lepidozona mertensii* (Middendorff, 1847) (SBMNH 83145 and 369438), *Lepidozona pectinulata* (Carpenter in Pilsbry, 1893) (SBMNH 83152 and 83153), *Placiphorella velata* Carpenter MS, Dall, 1879 (SBMNH 83161 and 369440), *Nuttallochiton hyadesi* (de Rochebrune, 1889) (SBMNH 83157), *Ischnochiton textilis* (Gray, 1828) (SBMNH 83158 and 369435), *Ischnochiton variegatus* (H. Adams and Angas, 1864) (SBMNH 83159 and 369437), and *Lepidopleurus cajetanus* (Poli, 1791) (SBMNH 83154 and 83155).

While most genera thought to belong in the Mopaliidae (plus a few other families) were included in this study, *Amicula* and *Cryptochiton* were not. *Cryptochiton* adults lack

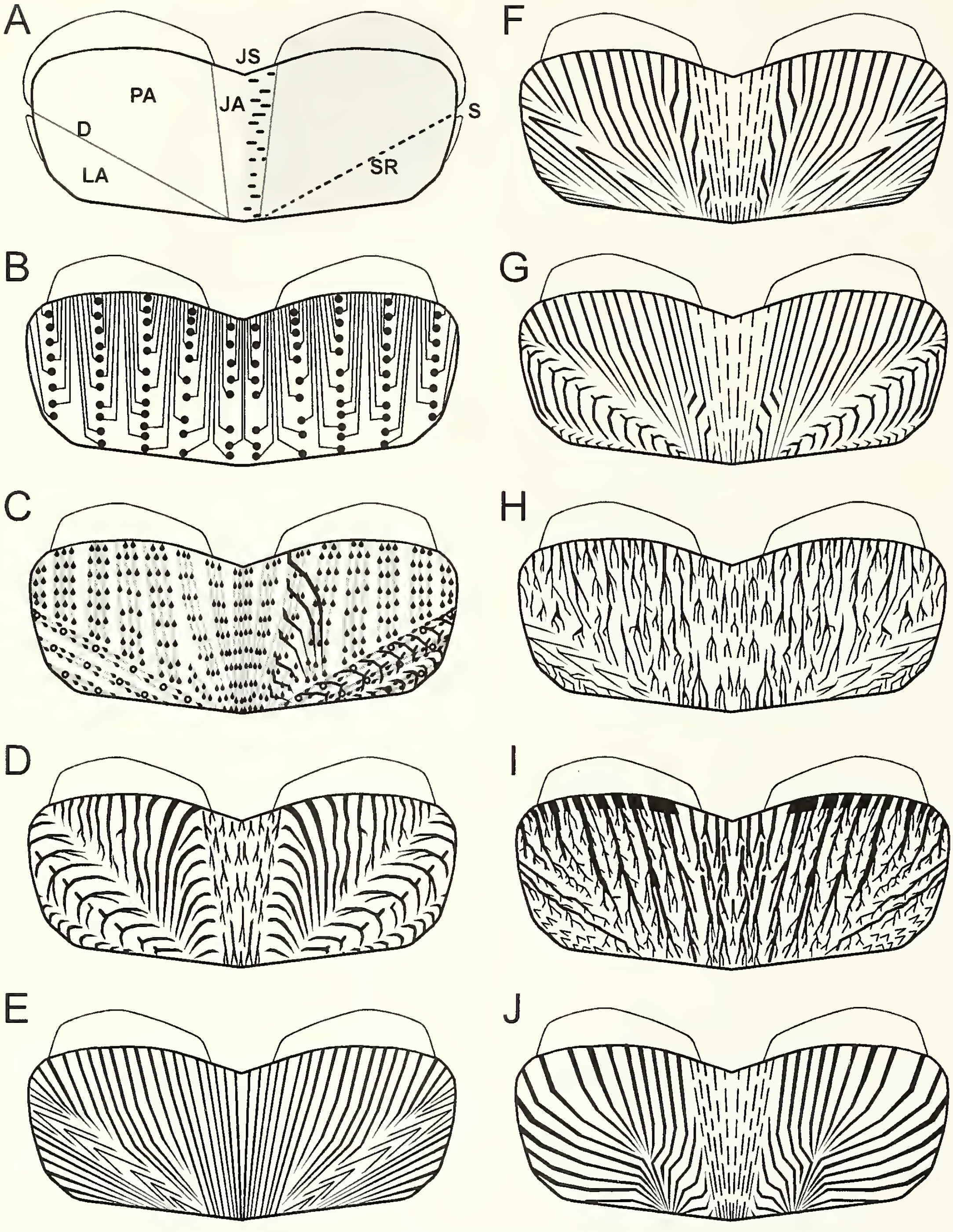

Figure 1. Schematic of the horizontal canal system in different chiton genera. A, Chiton intermediate valve showing dorsal (left side) and ventral (right side) features and terminology: D, diagonal line; JA, jugal area or jugum; JS, jugal sinus; LA, lateral area; PA, pleural area (also referred to as median triangle (Baxter and Jones 1981) or median area (Baxter and Jones 1984)); S, slit; SR, slit ray. B, Schematic of horizontal canals (lines) and megalaesthetes (filled circles) in *Lepidopleurus*, based on the pattern seen in *L. cajetanus*. C, *Lepidozona*, based on *L. cooperi*, *L. pectinulata*, and *L. mertensii*. D, *Ischnochiton*, based on *I. textilis* and *I. variegatus*. E, *Mopalia* type 1, characterizing *M. acuta*, *M. muscosa*, and *M. lignosa*. F, *Mopalia* type 2, characterizing *M. ciliata*, *M. spectabilis*, and *M. swanii*. G, *Tonicella*, based on *T. lokii*, *T. lineata*, *T. insignis*, and *T. marmorea*. H, *Plaxiphora*, based on *P. aurata*. I, *Cyanoplax*, based on *C. hartwegii*. J, *Nuttallina*, based on *N. californica*. The reconstructions in I and J were based on aesthete casts of *Cyanoplax hartwegii* (SBMNH 83146, 83147) and *Nuttallina californica* (SBMNH 83148, 83149, 83156) shown in Fernandez *et al.* (2007).

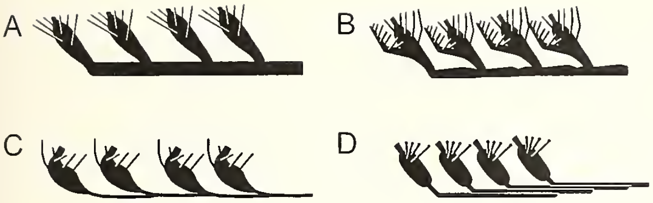

Figure 2. Schematic showing comparative morphology of canals that extend from the dorsal valve surface to the underlying horizontal canals. A, Form characteristic of *Mopalia* spp., *Tonicella* spp., *Dendrochiton* spp., *Placiphorella velata*, and *Katharina tunicata*. B, Form characteristic of *Cyanoplax hartwegii*, *Nuttallina californica*, *Plaxiphora aurata*, and *Nuttallochiton* spp. C, Form characteristic of *Ischnochiton* spp., *Tonicia chilensis*, and *Lepidozona* spp. D, Form characteristic of *Lepidopleurus cajetanus*. Reconstructions of *C. hartwegii*, *N. californica*, *P. aurata*, *Nuttallochiton*, *Ischnochiton*, and *Lepidozona* are based on aesthete canal casts described and photographed in Fernandez *et al.* (2007).

a tegmentum, and adults of *Amicula* have only a small remnant of that shell layer, which limits the extent to which their aesthete canal systems can be compared to those of other mopaliids. Valves of juvenile *Cryptochiton stelleri* Middendorff, 1847 have some tegmentum, but we were not able to obtain juveniles of this species for destructive analysis.

The cladistic analysis using only aesthete canal characters was constructed with PAUP 4.0b10 (Swofford 2002). All taxa from Fernandez *et al.* (2007) as well as those herein ($N = 26$ total from both studies) were scored for the analysis, although five taxa had the same exact character states as another taxon in the analysis, so these "redundant" taxa were excluded ($N = 21$ in this analysis) to allow for branch-and-bound analysis over a reasonable time frame. Specifically, *Mopalia lignosa* had the same character states as *Mopalia ciliata*, *Mopalia spectabilis* had the same as *Mopalia muscosa*, *Tonicella marmorea* had the same as *Tonicella lineata*, *Tonicella lokii* had the same as *Tonicella insignis*, and *Lepidozona cooperi* had the same as *Lepidozona mertensii*. All characters were un-weighted and all character states unordered (description of characters and their states in Appendix 2). *Lepidopleurus cajetanus* was used as the outgroup. A branch-and-bound search was completed using maximum parsimony.

All epoxy casts and voucher shell plates from each individual in this study as well as those in the previous one (Fernandez *et al.* 2007) have been deposited at the SBMNH.

RESULTS

Reference to the trend of the canal system in the descriptions to follow is consistent with the flow of sensory information and the direction of valve growth (see Baxter and Jones 1981, 1984), such that the pores on the dorsal tegmentum surface are taken to be the entrance and the sites where the canals enter the body of the chiton (large pores in the anterior and lateral tegmentum eaves, slit rays, and underneath the jugum) the exit. The terms anterior, posterior, dorsal, and ventral refer to the valve in life position. The two pieces of the aesthete canal cast are termed dorsal and ventral, also defined based on life position.

A nearly complete cast of the aesthete canal system was achieved in most relatively un-eroded valves. The few eroded valves (*e.g.*, from one individual of *Plaxiphora aurata*), in contrast, had missing canals and a high incidence of tunnels caused by endolithic organisms. The ventral casts in this study often had at least a few complete vertical canal elements (*i.e.*, extending from the dorsal to ventral surface of the valve), allowing a detailed examination of the megalaesthete-micraesthete complex in certain portions of the valve. The results reveal variation in the horizontal canal system (Fig. 1) as well as megalaesthete-micraesthete morphology (Fig. 2).

Data from the aesthete canal casts of *Mopalia acuta*, *Mopalia muscosa*, and *Nuttallochiton hyadesi*, described in Fernandez *et al.* (2007), are also incorporated into the following descriptions of the canal system in each genus.

The taxonomic assignments are based on Sirenko (1997, 2006) but assignments that differ between Sirenko (2006) and what is suggested by the phylogeny in Eernisse (unpubl. data) are indicated with a question mark. Specifically, Eernisse's phylogenetic hypothesis suggests *Dendrochiton* and *Tonicella* are in the Mopaliidae and *Nuttallochiton* is not.

Mopalia (Acanthochitonina: Mopaliidae) (Figs. 3, 4E-I)

The dorsal casts reveal large (ranging from about 20-75 μm diameter), nearly straight, roughly equal diameter, regularly spaced, primary horizontal canals that run from the posterior to anterior margin through all valve areas (Figs. 3C, 3E, 3H, 4E, 4I; also fig. 2a,b,j in Fernandez *et al.* (2007)). The canals are closely spaced (about 10-40 μm between primary canals) and only rarely do they merge with each other. At least two vertical levels of primary horizontal canals can be seen at their exit near the anterior margin of the valve. In *Mopalia ciliata*, *Mopalia spectabilis*, and *Mopalia swanii*, the horizontal canals on either side of the jugal area fan out laterally (Figs. 3H, 4A, 4I), whereas in the other species of *Mopalia*, the long axes of all horizontal canals in the central area are consistently straight (Fig. 3C; also fig. 2a,j in Fernandez *et al.* (2007)).

There are also many short, smaller-diameter (about 10-20 μm) subsidiary horizontal canals, above (in life) and inclined relative to primary horizontal canals, that merge with the primary canals at regular intervals (Figs. 3A-B, 3D, 4G;

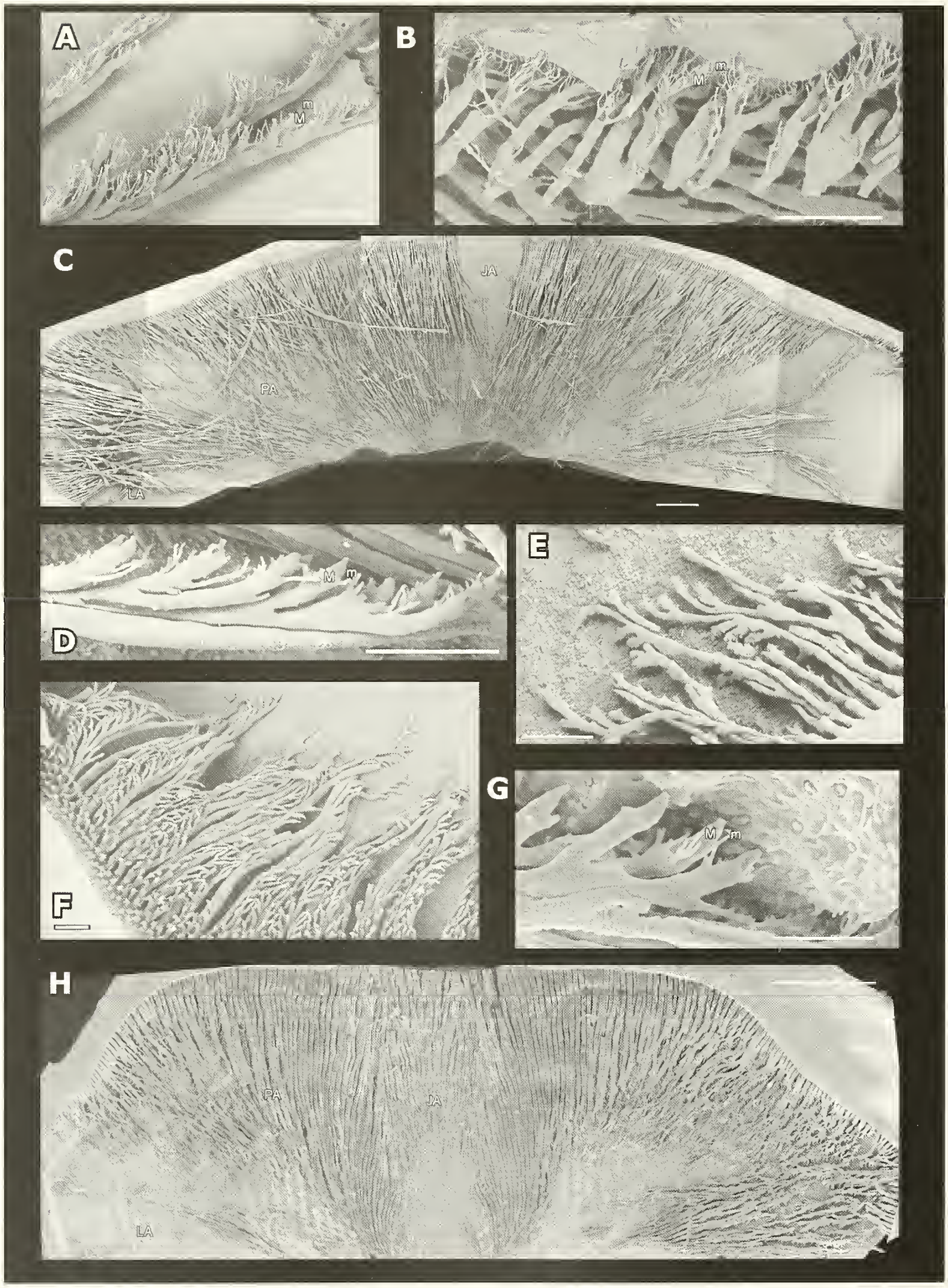

Figure 3. SEM images of casts of aesthete canal systems for *Mopalia spectabilis* (SBMNH 369491) (A-B), *Mopalia lignosa* (C-E), and *Mopalia swanii* (SBMNH 83329) (F-H). All images are of dorsal casts, except A which is of the ventral cast. A-B, *Mopalia spectabilis*. A, Close-up of a complete slit ray canal element on the ventral cast. Scale bar = 200 µm. B, Close-up of lateral area canals along the posterior margin of a different individual than in A. Scale bar = 200 µm. C-E, *Mopalia lignosa*. C, Composite image showing view of much of the system of horizontal canals (SBMNH 83328). Scale bar = 1 mm. D, Close-up of canals in the pleural area (SBMNH 83328). Scale bar = 200 µm. E, Canals in the pleural area along the anterior margin of a different individual (SBMNH 83327). Scale bar = 200 µm. F-H, *Mopalia swanii*. F, Complete canals in the pleural area. Cast was made by draining epoxy off valve prior to curing. Scale bar = 200 µm. G, Close-up of canals in the pleural area. Scale bar = 100 µm. H, Composite image showing horizontal canal pattern. Scale bar = 1 mm. Key: JAc, jugal area channel; M, megalaesthete chamber; m, micraesthete canal; SRc, slit ray channel; all other abbreviations as in Fig. 1A.

also fig. 2i in Fernandez *et al.* (2007)). Gently expanding megalaesthete chambers connect to these subsidiary canals (Figs. 3A, 3D, 3G, 4F-H; also fig. 2c,i in Fernandez *et al.* (2007)). Megalaesthete chambers begin as a short length of canal with a diameter of about 12-15 µm, before gently flaring out as they continue down towards the horizontal canals. At least four micraesthete canals (about 2-4 µm in diameter) trend in a straight, slightly angled to vertical manner to enter each megalaesthete chamber where it first reaches maximum diameter (Fig. 4F). At the top of the casts of the micraesthete canals and megalaesthete chambers are cup-shaped protuberances (Figs. 3G, 4G) that appear to be casts of subsidiary and apical caps, respectively.

In the jugal area of dorsal casts, some horizontal canals have a more flattened appearance and project upward (*i.e.,* turn down towards the ventral valve surface in life). The canals that exit at, or very close to, the jugal sinus, on the other hand, have a circular cross-section. On the ventral casts, the corresponding area (referred to as the ventral jugal triangle in Fernandez *et al.* (2007)) has short lengths of similarly flattened canals. These ventral canals occur in rows that correspond to valve growth lines; about five or more canals along some growth lines can be seen on the casts of species such as *Mopalia ciliata*. Most of the *Mopalia* spp. have a large number of canals that exit below the jugum, but *Mopalia acuta* has only very few jugal area canals that exit ventrally.

In *Mopalia ciliata*, *Mopalia spectabilis*, and *Mopalia swanii*, the horizontal components of the slit ray canals occur through a large portion of the lateral areas (Figs. 4E, 4I), with canals on either side of the slit ray progressing at a low angle relative to each other to meet and then turn downwards towards the slit ray. In contrast, the other species of *Mopalia* have slit ray canals that only extend for a short distance to either side of the slit ray, with the two horizontal components meeting at an even lower angle (Fig. 3C; also Fig. 2a,j in Fernandez *et al.* (2007)).

Tonicella (Acanthochitonina: Tonicellidae?) (Figs. 4A-D, 5)

This genus has large (ranging from about 40-75 µm diameter), long, somewhat wavy, wide, very regularly spaced, main horizontal canals that occur from the anterior to posterior margin of the valves (Figs. 4A, 5A-B, 5F, 5L). The spacing of canals is even more regular than in the *Mopalia* spp., giving the aesthete canal system in this genus the most orderly appearance. The main horizontal canals regularly meet gently-tapering megalaesthete chambers (*e.g.,* Fig. 4B, 4D, 5E, 5G, 5I) that are themselves embedded with numerous micraesthete canals. These megalaesthete chambers are connected obliquely downward by short canals to the main horizontal canals. The micraesthetes tend to be relatively short and straight.

The jugal area canals begin as micraesthetes that connect to gently tapering megalaesthete bulbs that connect to long, occasionally somewhat sprawling (Fig. 4H), canals that after a short distance begin to turn downward. These canals extend for a fair distance before merging with others into a larger canal, which then extends for a distance before merging with an even larger one (Fig. 5J). All the species in this genus had a high number (>30) of canals exiting ventrally below the jugum.

The slit ray canals make up the entire lateral area of the tonicellids, and have a high arc, with a consistent curve towards each other (Figs. 4A, 4C, 5A, 5C, 5F, 5L). On the ventral casts, the slit ray exits can be seen to begin as a line near the apex, but often split up into two or more rows towards the lateral margins of the valve.

Katharina (Acanthochitonina: Mopaliidae) (Figs. 6D-G)

The dorsal casts of *Katharina tunicata* reveal straight, regularly spaced, fairly large (about 30-40 µm diameter), densely packed horizontal canals that are angled towards the posterior apex in the lateral and pleural areas. The jugal area is dominated by canals that exit ventrally ("jugal area channels" of Baxter and Jones (1981)) (Fig. 6F). The jugal area canals have a high rate of merging before exiting at the ventral surface below the jugum. The morphology of the canals that lead into these horizontal canals is quite variable within the two individuals observed. In most cases, the micraesthetes connect to gently tapering megalaesthete chambers that then connect through a short canal into a main horizontal canal. In other cases, a large number of micraesthetes connect to long, narrow, often branching canals that then lead to a main horizontal canal or into a megalaesthete chamber (Fig. 6D). In the jugal area channels, numerous long, straight micraesthetes merge into the large canals either directly or by first merging into short, intermediate sized canals (Fig. 6E).

The apical area canals are quite noticeable on the ventral cast (Fig. 6G), perhaps because the apical area is more extensive in this species than in any other in the study. Many large horizontal canals occur in the apical area, connecting to micraesthetes that originate on the ventral or posterior surface of the apical area. The slit ray canals were not clearly seen in the SEM images of the casts.

Dendrochiton (Acanthochitonina: Tonicellidae?) (Figs. 7A-F)

The dorsal casts show large (about 40-50 µm diameter), long, fairly dense (about 60 µm between adjacent canals), somewhat wavy main horizontal canals that occur from the anterior to posterior margin of the valve (Figs. 7A, 7E). These canals have regular intersections with gently expanding megalaesthete chambers (Figs. 7C-D, 7F). Numerous straight micraesthetes trend downward and attach at the megalaesthete chamber all along its extent nearly up to its

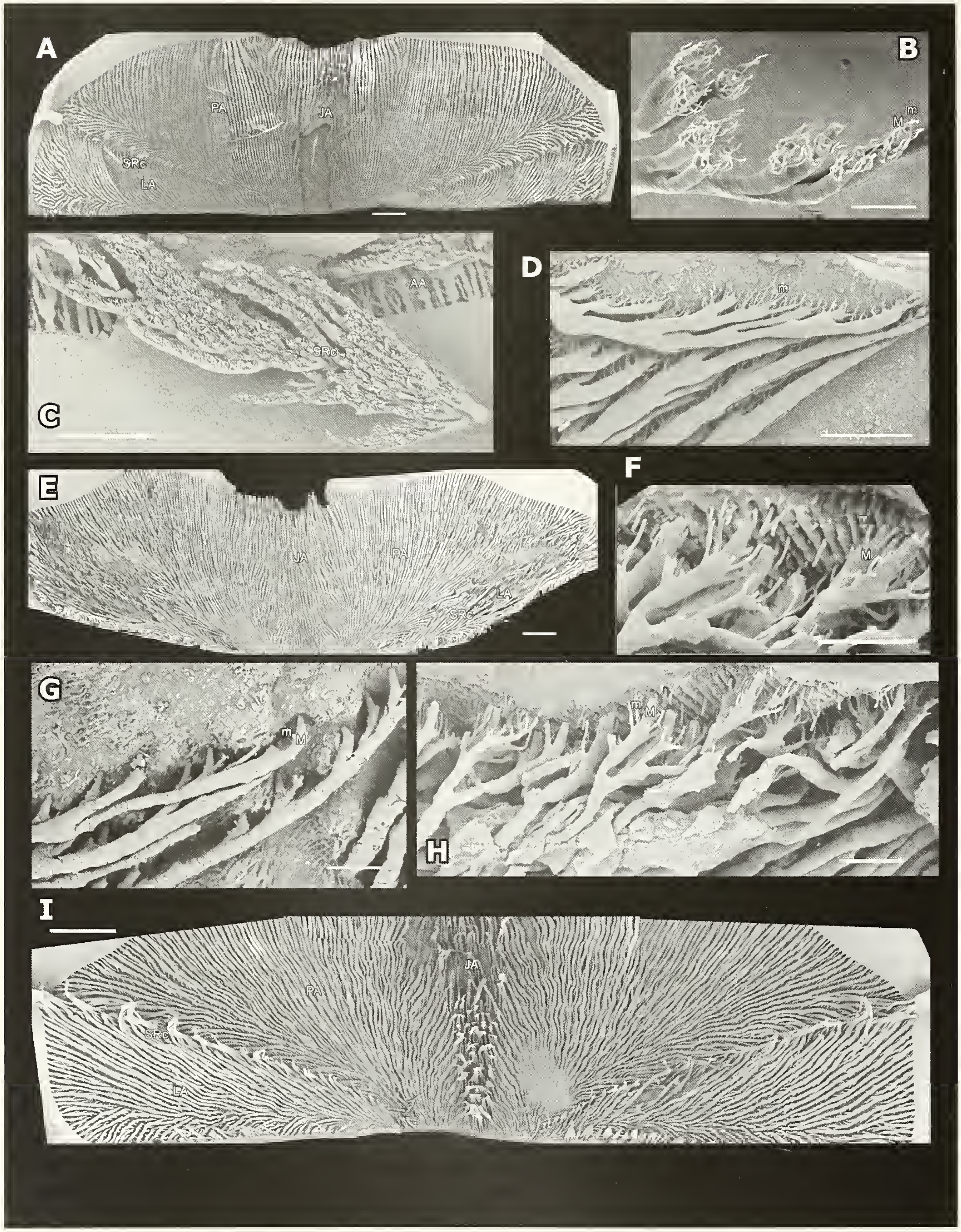

Figure 4. SEM images of casts of aesthete canal systems for *Tonicella insignis* (SBMNH 369497) (A-D), *Mopalia ciliata* (SBMNH 369501) (E-H), and *Mopalia spectabilis* (SBMNH 369501) (I). B and C are photos of ventral casts; all others are dorsal casts. A-D, *Tonicella insignis*. A, Composite image showing the whole horizontal canal pattern. Scale bar = 1 mm. B, Close-up of the near-surface portion of a slit ray canal. Scale bar = 100 µm. C, View of slit ray canals overlying (in this image) apical area canals in a different individual than shown in A-B. Scale bar = 200 µm. D, Close-up of the lateral area near the posterior margin of the valve. Scale bar = 200 µm. E-H, *Mopalia ciliata*. E, Composite image showing the entire horizontal canal system (SBMNH 83326). Scale bar = 1 mm. F, View of canals along the posterior margin (in between the lateral area and apical area) (SBMNH 83325). Scale bar = 100 µm. G, Close-up of canals in lateral area (SBMNH 83325). Scale bar = 100 µm. H, Close-up of canals in jugal area (SBMNH 83325). Scale bar = 100 µm. I, *Mopalia spectabilis*, composite image showing the whole horizontal canal pattern. Scale bar = 1 mm. Key: AA, apical area; others as in Figs. 1A and 3.

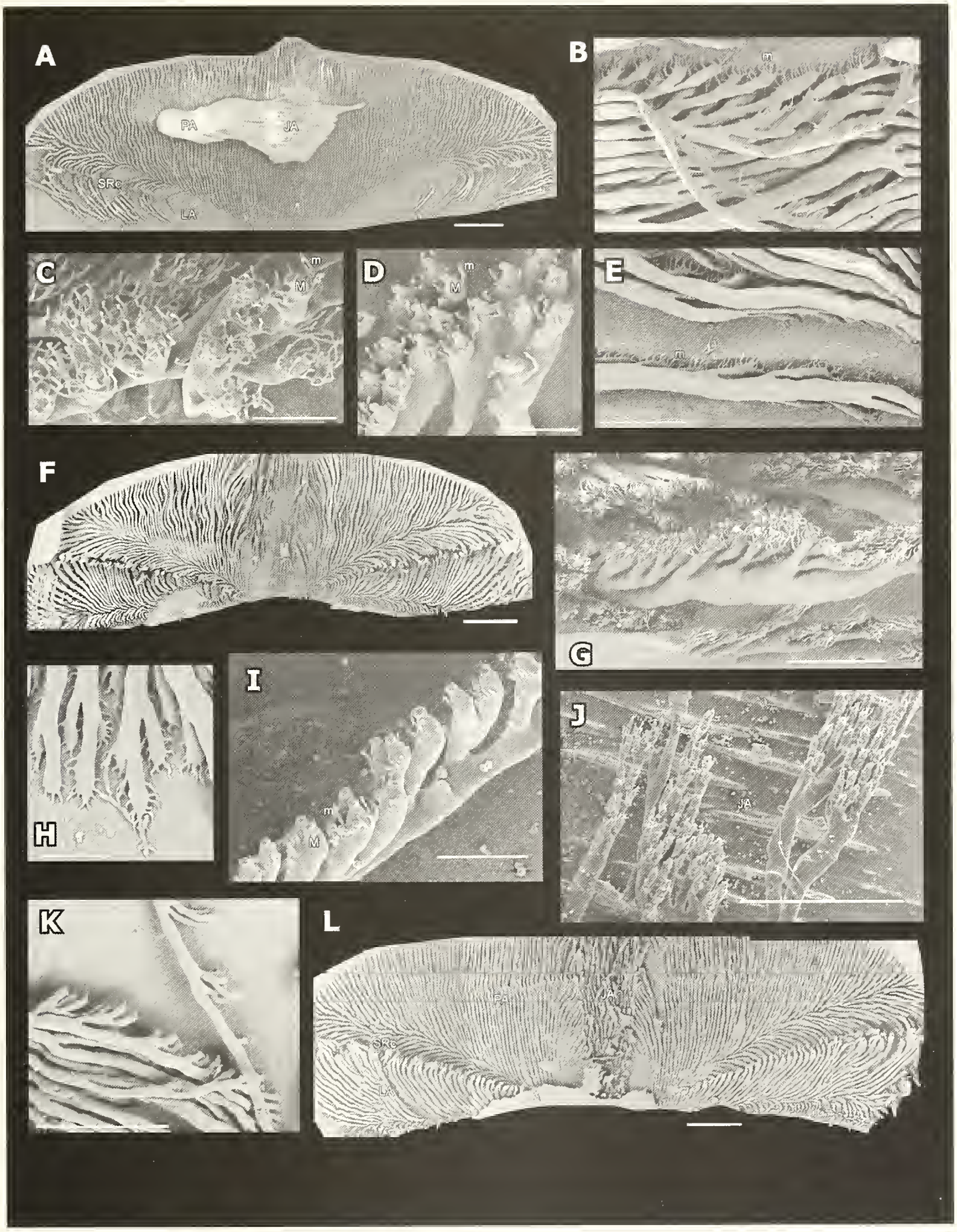

Figure 5. SEM images of casts of aesthete canal systems for *Tonicella marmorea* (SBMNH 369496) (A-D), *Tonicella lineata* (SBMNH 369488) (E-H), and *Tonicella lokii* (I-L). All images are of dorsal casts, except C, D, G, J, and I, which are images of ventral casts. A-D, *Tonicella marmorea.* A, Composite image showing complete horizontal canal system, with some remnant shell material in the middle. Scale bar = 1 mm. B, Close-up of region of pleural area with some horizontal canals scraped aside. Scale bar = 200 µm. C, Close-up of dorsal portion of slit ray canals. Scale bar = 100 µm. D, Close-up of some canals in the apical area. Scale bar = 100 µm. E-H, *Tonicella lineata.* E, Close-up of canals in the pleural area, with some missing adjacent horizontal canals. Scale bar = 200 µm. F, Composite image showing overall horizontal aesthete canal pattern. Scale bar = 1 mm. G, Close-up of canals in the apical area. Scale bar = 200 µm. H, Close-up of jugal area canals. Scale bar = 100 µm. I-L, *Tonicella lokii.* I, Close-up of dorsal portion of slit ray canals on the ventral cast (SBMNH 83319). Scale bar = 100 µm. J, Close-up of jugal area canals on the ventral cast (SBMNH 83319). Scale bar = 500 µm. K, Close-up of canals in the pleural (right) and lateral (upper left) region of the shell, with numerous horizontal canals missing, dorsal cast (SBMNH 83319). Scale bar = 500 µm. L, Composite image showing the overall horizontal aesthete canal pattern of a different individual (SBMNH 83320). Scale bar = 1 mm. Key: same as in Figs. 1A and 3.

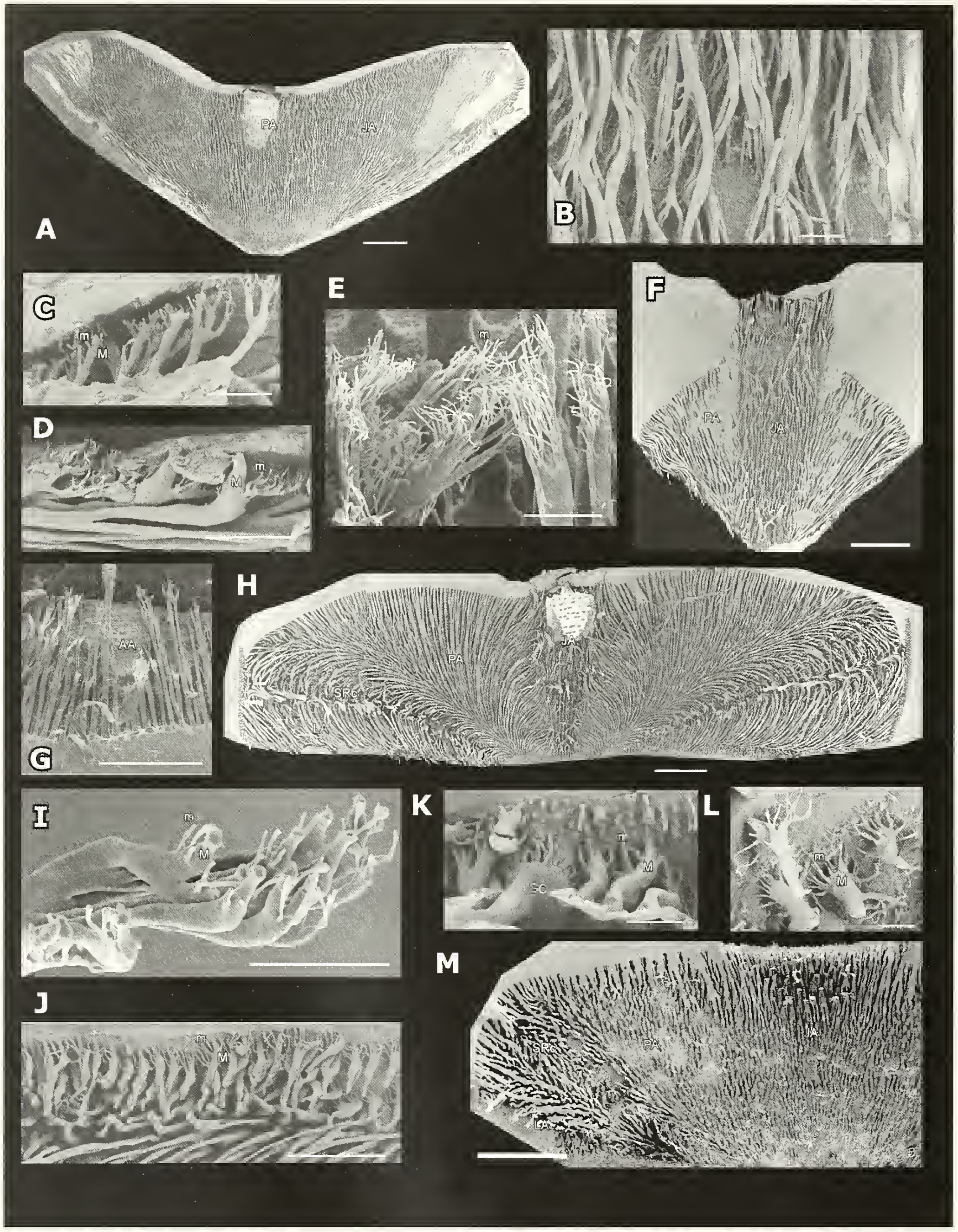

Figure 6. SEM images of casts of aesthete canal systems for *Plaxiphora aurata* (SBMNH 83321) (A-C), *Katharina tunicata* (SBMNH 369494) (D-G), *Tonicia chilensis* (SBMNH 369486) (H-K), and *Cyanoplax hartwegii* (SBMNH 83147) (L-M). Photos E, G, and I are of ventral casts; all others are of dorsal casts. A-C, *Plaxiphora aurata*. A, Composite image showing pattern of the horizontal canal system. Scale bar = 1 mm. B, Close-up of jugal area canals. Scale bar = 100 μm. C, Close-up of canals in the lateral area, near the posterior margin. Scale bar = 100 μm. D-G, *Katharina tunicata*. D, Close-up of canals in the lateral area, along the posterio-lateral margin. Scale bar = 100 μm. E, Close-up of dorsal portion of jugal area canals. Scale bar = 1 mm. F, Composite image showing horizontal canal system. G, Close-up of canals in the apical area. Scale bar = 500 μm. H-K, *Tonicia chilensis*. H, Composite image showing horizontal canal system. Some remnant shell material visible in middle anterior. Scale bar = 1 mm. I, Close-up of slit ray canals. Different individual than in H, J-K. Scale bar = 100 μm. J, Close-up of lateral area along the posterior margin. Scale bar = 200 μm. K, Close-up of pleural area showing large chamber (that presumably contained an ocellus). Scale bar = 50 μm. L-M, *Cyanoplax hartwegii*. L, Close-up of lateral area canals at the posterio-lateral corner. Scale bar = 100 μm. M, Composite image showing horizontal canal pattern. Scale bar = 1 mm. Key: GC, giant chamber, presumably that held an ocellus; others as in Figs. 1A, 3, and 4.

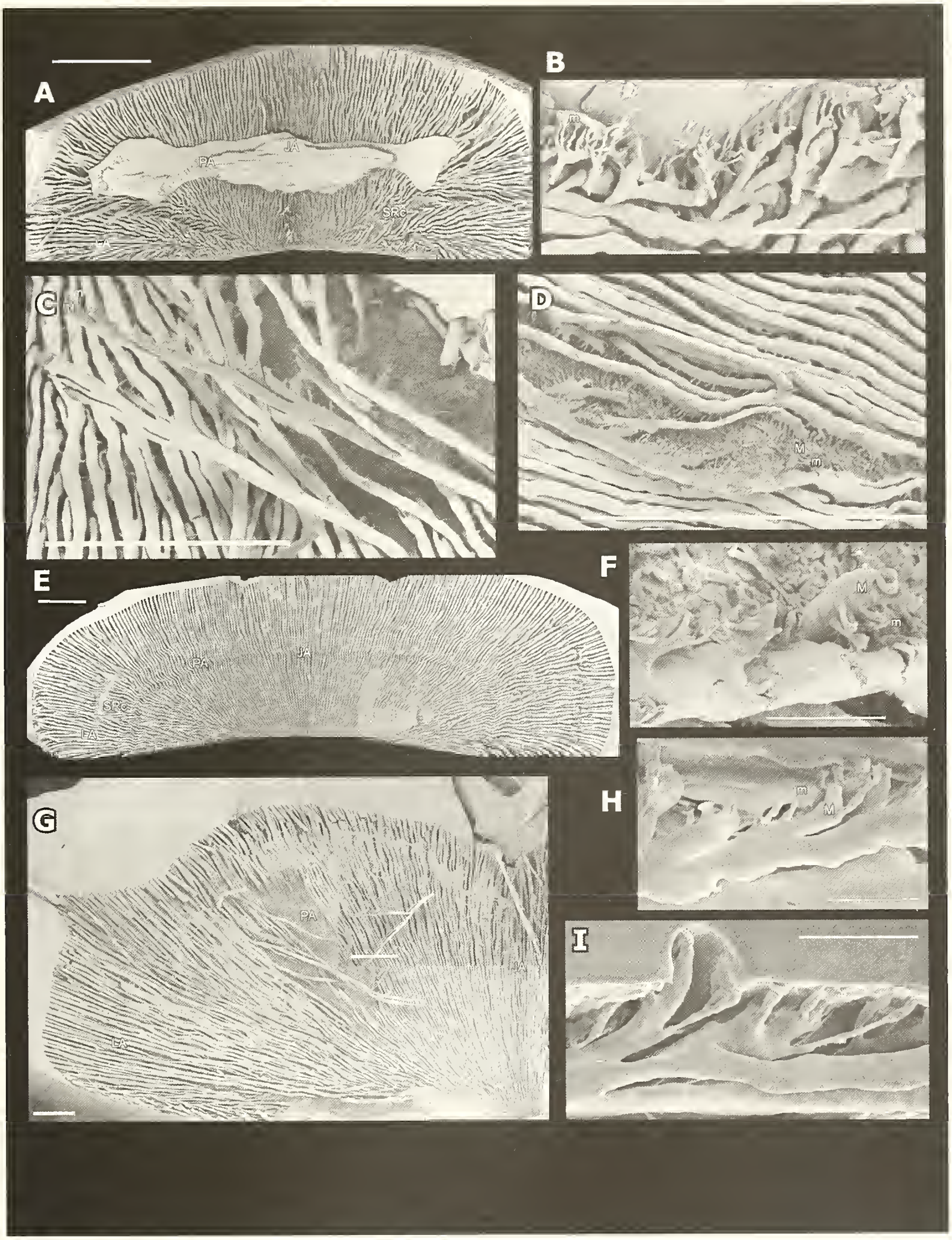

Figure 7. SEM images of casts of aesthete canal systems for *Dendrochiton lirulatus* (SBMNH 369493) (A-C), *Dendrochiton flectens* (SBMNH 369492) (D-F), and *Nuttallochiton mirandus* (SBMNH 83324) (G-I). All images are of dorsal casts. A-C, *Dendrochiton lirulatus*. A, Composite image showing view of much of the system of horizontal canals, with some remnant shell material in the center. Scale bar = 1 mm. B, Close-up of the lateral area near posterior margin. Scale bar = 200 µm. C, View of pleural area, portion of anterior margin in upper right. D-F, *Dendrochiton flectens*. D, View of pleural area showing a region with horizontal canals pulled aside, showing megalaesthete and micraesthete canals. Scale bar = 1 mm. E, Composite image showing view of the complete horizontal canal system. Scale bar = 1 mm. F, Close-up view of region of D. Scale bar = 100 µm. G-I, *Nuttallochiton mirandus*. G, Composite image showing one half of the entire horizontal canal system. Scale bar = 1 mm. H, View of jugal area surface canals along the broken margin. Scale bar = 100 µm. I, View of another region of jugal area surface canals along the broken margin. Scale bar = 100 µm. Key: same as in Figs. 1A and 3.

intersection with the main horizontal canal (Figs. 7B, 7D, 7F).

The jugal area channels are more prominent in *Dendrochiton lirulatus* than in *Dendrochiton flectens* (compare Figs. 7A and 7E). The jugal area channels begin as micraesthetes that connect to sprawling, sub-cylindrical, horizontal canals that then connect to cylindrical canals that turn downwards, merging with others of their kind, before terminating at the ventral valve surface below the jugum.

In one individual of *Dendrochiton flectens*, canals exited at many different places on the ventral surface of the valve, not just below the jugum and in the slit rays. Such a pattern has not been seen in any of the twelve species studied by Fernandez *et al.* (2007) or in the thirteen other species in this study, and likely resulted from abnormal growth. The horizontal components of the slit ray canals have a relatively narrow lateral extent (Figs. 7A, 7E) and form a single prominent line of pores that make up the slit ray.

Plaxiphora (Acanthochitonina: Mopaliidae?) (Figs. 6A-C)

This species is characterized by micraesthete canals that enter small narrow canals or gently tapering megalaesthete chambers (Fig. 6C) that then connect with small horizontal canals (about 15-20 µm diameter), which may merge a few times until becoming relatively narrow, widely spaced, wavy main horizontal canals (Fig. 6B). One individual examined, whose valves were eroded, had a high density of tunnels made by endolithic organisms. There are very few jugal area canals apparent on either the dorsal or ventral casts of either specimen.

The horizontal components of the slit ray canals extend for a short width on either side of the slit ray, meeting at a low angle (Fig. 6A), nearly sub-parallel to the slit ray, before the merged canal trends upward on the dorsal cast (downward in life) towards pores along the slit ray.

The apical area canals, seen on the ventral cast, show relatively widely-spaced horizontal canals running most of the length of the apical area. They originate as micraesthete canals on the ventral surface of the apical area, near or along the posterior margin. These small-diameter canals widen and then, in many cases, merge with another horizontal canal as they progress toward the anterior margin of the apical area.

Nuttallochiton (Acanthochitonina: Mopaliidae?)
(Figs. 7G-I)

The dorsal casts reveal primary horizontal canals (about 30-50 µm in diameter) throughout the entire interface between the tegmentum and articulamentum (Fig. 7G; also fig. 2g in Fernandez *et al.* (2007)). There is a spacing of about 20-50 µm between canals, with about 18 canals per mm along the horizontal plane. Micraesthetes (1-3 µm diameter) connect to the elongate, indistinct megalaesthete chambers that regularly connect, after a short distance, to the primary horizontal canals (Fig. 7H; also fig. 2h in Fernandez *et al.* (2007)). The megalaesthete chambers have a diameter of about 10-12 µm before widening to the same diameter as the connecting canals (about 17-20 µm).

Nuttallochiton mirandus appears to have no, or very few, jugal area canals and very few slit ray canals. The latter condition contrasts with *Nuttallochiton hyadesi*, which has a wider lateral extent of the horizontal components of the slit ray canals (compare Fig. 7G with fig. 2g in Fernandez *et al.* (2007)).

Tonicia (Chitonina: Chitonidae) (Figs. 6H-K)

The specimens of *Tonicia chilensis* show relatively narrow (about 30-40 µm diameter), widely spaced (about 50-100 µm between canals), curving horizontal canals that repeatedly intersect canals from megalaesthete chambers. All the megalaesthete chambers consist of bulbs that are embedded with a relatively small number of micraesthete canals (Figs. 6I-J). The main horizontal canals on either side of the jugal area have a very high arc towards the lateral margin. In a few locations, extremely large aesthete bulbs occur (much larger than the typical megalaesthete chambers), that are embedded with an immense number of micraesthete canals (Fig. 6K).

The jugal area canals are abundant and cover most of the valve surface (Fig. 6H). Many of these canals originate in the pleural areas and may even extend to the lateral areas. Such a wide extent of jugal area canals has not been seen in any of the twelve species examined in Fernandez *et al.* (2007) or in any of the other thirteen species in this study. The slit ray canals take up the entire lateral area (Fig. 6H) and have a similar degree of convergence of canals as in the jugal area channels of the central area.

DISCUSSION

Variation in aesthete canal characters

The results provide further evidence for variation in aesthete canal morphology among chiton suborders, families, genera, and often species (Table 1). Building from Fernandez *et al.* (2007), the results herein confirm that many chiton taxa at all taxonomic ranks are unified by synapomorphies (whether a character state is primitive or derived, assessed by the cladistic analysis using *Lepidopleurus cajetanus* as an outgroup) and that aesthete features have considerable potential as phylogenetic characters at a number of levels.

The cladistic analysis herein (Fig. 8) yields a phylogenetic hypothesis based solely on the broad morphology of the aesthete canal system across a large group of chitons, mostly within the Suborder Acanthochitonina. The cladistic analysis is meant to: (1) show the similarities and differences in the aesthete canal system between a larger set of chitons; (2) refine characters and character states from Fernandez *et al.* (2007), in light of the new information, to make the characters/states more useful for future phylogenetic analyses of a broader range of taxa; and (3) test a recent view of mopaliid phylogeny (Eernisse, unpubl data).

Many other aspects of chiton morphology have been used in phylogenetic studies of chitons, including egg hull characters (Eernisse 1984, 1988, Sirenko 1993, 1997, 2006), sperm morphology (Hodgson *et al.* 1988, Buckland-Nicks 1995, 2006, Buckland-Nicks and Hodgson 2000), radula and radular tooth biomineralization patterns (Bullock 1985, Brooker and Macey 2001, Saito 2003), gill placement characters (Eernisse 1984, Sirenko 1993, 1997, 2006), and girdle and gland characters (Sirenko 2006). Moreover, Okusu *et al.* (2003) have been successful in using molecular sequences to infer chiton phylogeny. Attempts to determine the phylogenetic relationships within the Polyplacophora should of course incorporate as many of these characters as possible (Sirenko 2006), and we would add aesthete canal morphology to this list.

Aesthete canal morphology in the Mopaliidae

Fernandez *et al.* (2007) described how the mopaliids in their study (*Mopalia muscosa*, *Mopalia acuta*, *Placiphorella velata*, and *Nuttallochiton hyadesi*—but see below) are characterized by wide, straight, closely spaced, primary horizontal canals that exist through much of the valve length, as well as regular merging of short, subsidiary branches from the upper tegmentum with these primary canals. The subsidiary branches connect with only slightly expanded megalaesthete "chambers" just below the valve surface that are embedded with a large number of micraesthete canals. This pattern was also seen in all the *Mopalia* spp., *Katharina tunicata*, and some others (see below) in this analysis, strengthening the hypothesis that such aesthete canal characters typify mopaliids.

In addition, the results of this study show how *Mopalia ciliata*, *Mopalia spectabilis*, and *Mopalia swanii* share aesthete canal characters that are absent in *Mopalia acuta*, *Mopalia lignosa*, and *Mopalia muscosa*, based on the observations that the former group has a wider range of the slit ray canals and a more fan-like arrangement of the horizontal canals that flank the jugal area than the latter group.

Katharina tunicata shows the typical mopaliid pattern of long, straight, horizontal canals with gently tapering mega-

Table 1. Aesthete characters used in the PAUP analysis. Descriptions of characters and character states provided in Appendix 2. Key to abbreviations: jug, number of canals that exit ventrally under jugum; lat, nature of slit ray canals in lateral area; lin, linear arrangement and orderly spacing of megalaesthete bulbs; mgc, types of megalaesthete chambers; hgc, huge aesthete chambers; deh, density of horizontal canals; shc, connection between surface and main horizontal canals; flj, divergence of horizontal canals flanking jugal area; hap, straight horizontal canals; reg, regular merging of short canals into main horizontal ones; lam, lateral merging of main horizontal canals.

Species	jug	lat	lin	mgc	hgc	deh	shc	flj	hap	reg	lam
Mopalia ciliata	1	2	0	0	0	1	1	2	1	1	0
Mopalia muscosa	1	2	0	0	0	1	1	0	1	1	0
Mopalia swanii	1	2	0	0	0	1	1	2	1	1	0
Mopalia acuta	1	2	0	0	0	1	1	0	1	1	0
Tonicella lineata	1	1	0	0	0	1	1	2	1	1	0
Tonicella insignis	1	1	0	0	0	1	1	2	1	1	0
Tonicia chilensis	1	0	0	2	1	0	1	0	1	0	0
Placiphorella velata	1	2	0	0	0	1	1	0	1	1	0
Dendrochiton flectens	?	2	0	0	0	1	1	0	1	1	0
Dendrochiton lirulatus	1	2	0	0	0	1	1	0	1	1	0
Katharina tunicata	1	2	0	0	0	1	1	1	1	1	0
Nuttallochiton mirandus	0	1	0	1	0	1	0	0	1	0	0
Nuttallochiton hyadesi	0	2	0	1	0	1	0	0	1	0	0
Plaxiphora aurata	0	3	0	1	0	0	0	0	0	0	1
Ischnochiton textilis	1	0	0	2	?	0	1	0	0	0	0
Ischnochiton variegatus	1	0	0	2	0	0	1	0	0	0	0
Nuttallina californica	1	3	0	1	0	0	0	0	0	0	1
Cyanoplax hartwegii	1	3	0	1	0	0	0	0	0	0	1
Lepidopleurus cajetanus	1	4	1	3	0	0	2	0	0	0	0
Lepidozona mertensii	1	0	1	2	1	0	1	0	0	0	0
Lepidozona pectinulata	1	0	1	2	1	0	1	0	0	0	0

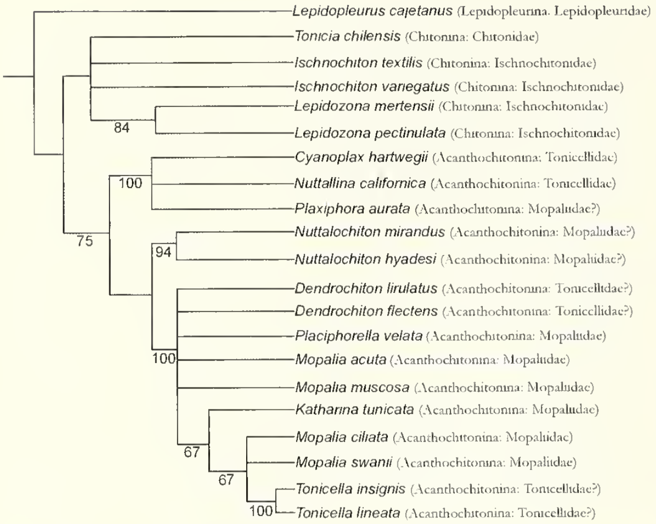
Lepidopleurus cajetanus (Lepidopleurina. Lepidopleuridae)
Tonicia chilensis (Chitonina: Chitonidae)
Ischnochiton textilis (Chitonina: Ischnochitonidae)
Ischnochiton variegatus (Chitonina: Ischnochitonidae)
Lepidozona mertensii (Chitonina: Ischnochitonidae)
Lepidozona pectinulata (Chitonina: Ischnochitonidae)
Cyanoplax hartwegii (Acanthochitonina: Tonicellidae)
Nuttallina californica (Acanthochitonina: Tonicellidae)
Plaxiphora aurata (Acanthochitonina: Mopaliidae?)
Nuttalochiton mirandus (Acanthochitonina: Mopaliidae?)
Nuttalochiton hyadesi (Acanthochitonina: Mopaliidae?)
Dendrochiton lirulatus (Acanthochitonina: Tonicellidae?)
Dendrochiton flectens (Acanthochitonina: Tonicellidae?)
Placiphorella velata (Acanthochitonina: Mopaliidae)
Mopalia acuta (Acanthochitonina: Mopaliidae)
Mopalia muscosa (Acanthochitonina: Mopaliidae)
Katharina tunicata (Acanthochitonina: Mopaliidae)
Mopalia ciliata (Acanthochitonina: Mopaliidae)
Mopalia swanii (Acanthochitonina: Mopaliidae)
Tonicella insignis (Acanthochitonina: Tonicellidae?)
Tonicella lineata (Acanthochitonina: Tonicellidae?)
84
100
75
94
100
67
67
100

Figure 8. Majority-rule consensus tree of the 96 most parsimonious trees (with 23 steps) that resulted from the cladistic analysis (PAUP) using only aesthete canal morphology. Numbers indicate majority rule consensus values. The data matrix for the analysis is shown in Table 1 and the characters and character states are listed in Appendix 2. The taxonomic assignments are based on Sirenko (1997 and 2006) but assignments that differ between Sirenko (2006) and what is suggested by the phylogeny in Eernisse (unpubl. data) are indicated with a question mark. Specifically, Eernisse's phylogenetic hypothesis suggests *Dendrochiton* and *Tonicella* are in the Mopaliidae and *Nuttallochiton* is not.

laesthete bulbs, but it also has some long, relatively narrow, horizontal, occasionally branching, subsidiary canals just below the surface that are embedded with numerous micraesthetes along their length, a character also seen in *Nuttallina californica* and *Cyanoplax hartwegii* (previously *Lepidochitona hartwegii*) (Fernandez *et al.* 2007).

Our previous study of chiton aesthete canal casts (Fernandez *et al.* 2007) revealed that *Nuttallochiton* bears strong similarities in the aesthete canal system with other members of the Mopaliidae, and the results of this study are not inconsistent with that interpretation. The cladistic analysis suggests that *Nuttallochiton* is either a basal group within the Mopaliidae or is the outgroup to that family (Fig. 8). Regardless of which interpretation is preferred, it is clear that the aesthete canal system of *Nuttallochiton* is intermediate between those of (other) mopaliids and other members of the Acanthochitonina such as *Cyanoplax* and *Nuttallina*. The *Nuttallochiton* species in this analysis and the previous one (Fernandez *et al.* 2007) share the large, closely spaced horizontal canals with mopaliids, but also share with *Cyanoplax* sprawling megalaesthete super-chambers that connect to the main horizontal canals via a canal subparallel to the surface, in addition to regular merging of short horizontal canals in the posterior half of the valve. However, *Nuttallochiton* differs from the members of the Acanthochitonina so far examined in lacking a large number of micraesthetes, and it differs from most other chitons so far examined in having no or very few canals that exit underneath the jugum. Overall, it shares similar aesthete canal characters both with the *Cyanoplax* group and undisputed members of Mopaliidae, but distinguishing between derived and plesiomorphic similarities will be best considered in the context of a more complete analysis of morphological and molecular evidence.

Plaxiphora had been historically placed in the Mopaliidae (*e.g.*, Kaas and Van Belle 1987, Sirenko 2006), but the results of this study suggest, as in Eernisse (unpubl. data), that this genus belongs outside of this family. *Plaxiphora* has a very high ratio of micraesthetes/megalaesthete, and a greater amount of shell material between neighboring horizontal canals than in the mopaliids. It also shares sprawling megalaesthete chambers with *Cyanoplax* and *Nuttallina*, other members of the Acanthochitonina, although a broader study incorporating more members of this suborder and the others is needed to better determine whether these shared characters are primitive or derived within this group. Regardless, *Plaxiphora* lacks the long, densely packed, straight main horizontal canals that characterize all mopaliids.

Tonicella shares many characters with the mopaliids (*e.g.*, large, closely-spaced, straight horizontal canals, same megalaesthete chamber shape, regular merging of short subsidiary canals with the primary horizontal canals). The results of the cladistic analysis are consistent with those of Eernisse (unpubl. data), which suggested *Tonicella* should be classified within the Mopaliidae. All four members of this genus analyzed in this study share remarkably similar aesthete canal systems (in particular, they have the most orderly arrangement of canals), and are each more similar to each other than any is to any of the other species whose aesthete canals have so far been described in detail.

The two species of *Dendrochiton* analyzed in this study share many aesthete characters with other mopaliids, such as the long, straight horizontal canals, non-descript shape of the megalaesthete bulbs, and a relatively large number of micraesthetes per megalaesthete (though not so many as in *Cyanoplax* and *Nuttallina*). Consistent with Kaas and Van Belle (1987), who listed *Dendrochiton* as a subgenus of *Lepidochitona*, the *Dendrochiton* species in this study share some characters of the aesthete canal system with *Cyanoplax hartwegii* and *Nuttallina californica*, such as the presence of long, narrow, horizontal canals that connect with megalaesthete chambers with many micraesthetes. However, *Dendrochiton* shares more synapomorphies with the mopaliids than it does with *Cyanoplax* and *Nuttallina*, consistent with the results of Eernisse (unpubl. data).

Dendrochiton and the other members of the Mopaliidae share many overall similarities in aesthete canal system with those of fellow members of the Acanthochitonina, *Cyanoplax*, and *Nuttallina*. These similarities include a high density of horizontal canals (though higher in mopaliids), high number of micraesthetes per megalaesthete (though higher in *Cyanoplax/Nuttallina*), and a regular merging of subsidiary canals with the main horizontal canal. In fact, the horizontal canal system drawn for *Lepidochitona cinerea* by Knorre (1925, fig. 37, as *Trachydermon cinereus*) is similar to that of mopaliids (Fig. 1F): relatively straight horizontal canals in the pleural area that extend most of the valve's length, with a regular merging of subsidiary canals, and with a relatively wide extent of the slit ray canals. Some of these similarities may provide evidence for grouping Mopaliidae and Lepidochitonidae (*e.g.*, *Lepidochitona*, *Cyanoplax*, *Nuttallina*) as a subclade within Acanthochitonina, or might be plesiomorphic features for Acanthochitonina, with differences noted in *Plaxiphora* and *Nuttallochiton* best considered derived features. Future studies of the aesthete canal system in other members of the Acanthochitonina, and the combination of these data with a wider range of morphological and molecular evidence should reveal which interpretation is more likely.

Conclusions

The results provide further evidence that characters of the aesthete canal system are phylogenetically informative at a number of taxonomic levels. This study approximately doubles the total number of comparisons possible, now 26 species, when these data are combined with those in the study by Fernandez *et al.* (2007). This present study is also significant in providing some of the first morphological evidence corroborating a new proposal based on molecular evidence. Specifically, variation in aesthete canal morphology is largely consistent with the new classification of the Mopaliidae proposed by Eernisse (unpubl. data), which excludes the genera *Plaxiphora* and *Nuttallochiton* from Mopaliidae (although the placement of *Nuttallochiton* is uncertain with respect to the Mopaliidae based on aesthete canal morphology alone) while including *Tonicella* and *Dendrochiton* within this family. Some characters in common between members of Mopaliidae and Lepidochitonidae could reflect synapomorphies for uniting these families within Acanthochitonina. More resolution is expected with the addition of other chiton species in future analyses of aesthete canal morphology, and the combination of these data with other morphological and molecular evidence will help elucidate relationships within Polyplacophora.

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Appendix 1. Collecting and locality information for the chitons used in this study.

Species	Accession #(s)	Collector(s)	Date collected	Locality notes
Dendrochiton flectens	SBMNH 369492	George Hanselman	1973	Underside of rocks during a –0.76 m tide, Cactus Island, Washington
Dendrochiton lirulatus	SBMNH 369493	George Hanselman	1971	Intertidal of Ensenada Blanca, Baja California Norte, Mexico
Katharina tunicata	SBMNH 83323	Michael Vendrasco	1999	Rocky intertidal, Cambria, California
Katharina tunicata	SBMNH 369494	George Hanselman	1972	Vancouver Island, British Columbia, Canada
Mopalia spectabilis	SBMNH 369491	Spencer Thorpe	1965	Morro Bay Harbor breakwater, California
Nuttallochiton mirandus	SBMNH 83324	Susanne Lockhart	2006	235 m depth, about 100 km south of Penguin Island, Antarctic
Plaxiphora aurata	SBMNH 83321-83322	Susanne Lockhart	2004	Intertidal, Tristan da Cunha, Sub-Antarctic
Tonicella lokii	SBMNH 83319-83320	Christine Fernandez and Michael Vendrasco	2006	Rocky intertidal, Cambria, California
Tonicella insignis	SBMNH 369497	Roger Clark	2000	Unalaska Island, Aleutian Islands, Alaska, 5-10 m depth
Tonicella lineata	SBMNH 369488	Spencer Thorpe	1965	Anacortes, Washington
Tonicella marmorea	SBMNH 369496	Ron McPeak	1977	Underside of rocks, 5-10 m depth, Seal Island, Nova Scotia, Canada
Tonicella marmorea	SBMNH 369495	Norm Curran	1964	Newagen, Maine
Tonicia chilensis	SBMNH 369486	Hank Chaney	2004	Under small rocks in tidepools, Cobija, Chile
Mopalia ciliata, *Mopalia lignosa*, and *Mopalia swanii*	SBMNH 83325-83330	George Hanselman	Unknown	California (additional details unknown)

Appendix 2. Description of aesthete characters and character states used in the cladistic analysis

1. Number of canals that exit ventrally under the jugum (jug): (0) 0-30, (1) >30.

Comments: area is the same as the "ventral jugal triangle" of Fernandez *et al.* (2007, fig. 1) and can be seen as the number of "jugal area channels" as defined in Baxter and Jones (1981, 1984). The number of canals in this area can be inferred from the number of pores seen on the ventral surface of valves in this region, the canal pieces in this region of the ventral cast, and in some cases in the dorsal cast, seen as upturned, typically flattened canals in the jugal area.

2. Nature of slit ray canals in lateral area (lat): (0) sparse and highly curved, (1) dense and highly curved, (2) dense and not highly curved, (3) sparse and not highly curved, (4) no slit ray canals.

Comments: refers to the extent of the horizontal portions of the slit ray canals that occur at the tegmentum/articulamentum interface. On the dorsal casts, these canals can be seen to merge parallel to the slit ray before trending downwards (in life; upwards on the dorsal cast) to a slit ray pore. This character is similar to character 8 (hcc: degree of horizontal canal curvature towards diagonal line) in Fernandez *et al.* (2007), although the divisions between character states are herein refined to match natural character state boundaries in the now larger taxon set.

3. Linear arrangement and orderly spacing of megalaesthete bulbs (lin): (0) absent, (1) present.

Comments: refers to well-organized anterior-posterior zones of megalaesthete chambers. This character can best be seen in some photos of *Lepidopleurus cajetanus* in Fernandez *et al.* (2007), which suggests that this character may be primitive in the crown group Polyplacophora. This character is the same as character 9 (apz: canals differentiated into anterior-posterior columns) in Fernandez *et al.* (2007).

4. Types of megalaesthete chambers (mgc): (0) type A, (1) type B, (2) type C, (3) type D.

Comments: the megalaesthete chamber types are illustrated in Fig. 7. Type A is a gently tapering chamber that only has a subtle bulb shape. Type B is a more sprawling chamber whose micraesthetes often merge before entering it. Type C is widest in the middle, with gradual tapering on both ends. Type D has an elongate form tapered sharply on both ends, like a sausage. Note this character refers to the typical shape of the megalaesthete chamber. Most species have at least some variation in the appearance of these chambers. Character 3 (blb: megalaesthete bulbs in central area) from Fernandez *et al.* (2007) makes up a portion of this newly expanded character.

5. Huge aesthete chambers (hgc): (0) absent, (1) present.

Comments: these are much larger than typical megalaesthete chambers and may be modified or merged megalaesthete chambers. These may contain ocelli. They are sparsely and apparently randomly distributed in *Tonicia* and are regularly distributed in large granules in *Lepidozona*. This character is similar to character 7 (hmc: huge aesthete chambers in large granules) in Fernandez *et al.* (2007), but is more broadly defined to allow the large chambers in *Tonicia* and *Lepidozona*—which appear homologous—to be coded the same.

6. Density of horizontal canals (deh): (0) very low (much visible space between canals), (1) low (some visible space between canals), (2) high (little visible space between canals).

Comments: refers to the density of primary horizontal canals at the tegmentum/articulamentum interface. This character is the same as that of the same number and code in Fernandez *et al.* (2007).

7. Typical connection between surface canals (*e.g.*, megalaesthete chambers) and main horizontal canals (shc): (0) long canal that is, in part, parallel to the surface, (1) short canal, oblique to surface, (2) each horizontal canal connects to only one megalaesthete bulb.

Comments: refers to the typical portion of the canal between the surface chambers and horizontal canals at the tegmentum/articulamentum interface. In *Lepidopleurus cajetanus*, each horizontal canal connects to only one megalaesthete bulb, making it difficult to compare with the other taxa (*i.e.*, it is difficult to say where the connecting canal "ends" and the horizontal canal "begins"). For this reason, *L. cajetanus* was coded as having a unique state (2).

8. Divergence of horizontal canals flanking the jugal area (flj): (0) absent, (1) tilted towards apex, (2) tilted away from apex.

Comments: refers to the set of main horizontal canals in the pleural area immediately adjacent to the jugal area. In some cases the angle of divergence (from the bisecting line) is high. Those canals that are tilted away from the apex are often arced and bend back towards the apex. This character is a modification/refinement of character 11 (doc: direction of convergence of horizontal canals in lateral area) in Fernandez *et al.* (2007).

9. Straight (or regularly wavy) horizontal canals from anterior to posterior margins (hap): (0) absent, (1) present.

Comments: this character can also be read as whether main (or primary) horizontal canals extend nearly the entire length of the valve.

10. Regular merging of short canals into main horizontal canals (reg): (0) absent, (1) present.

Comments: refers to whether there is a high rate of merging of obliquely-oriented, connecting canals from the megalaesthete chambers along the length of the main horizontal canals.

11. Lateral merging of main horizontal canals (lam): (0) absent, (1) present.

Comments: refers to a high rate of lateral merging of main horizontal canals, especially in the posterior portion of the valve.

General comments about characters: many of the characters used in Fernandez *et al.* (2007) were modified herein (see above) and some were excluded from this analysis. In some cases character states were modified to better match natural boundaries in the now larger taxon set. Character 1 from Fernandez *et al.* (2007) (agc: aesthete/granule correlation) was excluded because granules in many of the species examined are often indistinct, making it difficult to assess homology. Character 2 from that paper (are: megalaesthete canal morphology/pattern differ by valve area) was not used because it correlates with character 2 (lat) in this analysis and we decided against indirect weighting of that character.

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Mopalia kennerleyi Carpenter, 1864, a forgotten species and its southern analogue *Mopalia ciliata* (Sowerby, 1840)*

Roger N. Clark[1]

Department of Invertebrate Zoology, Santa Barbara Museum of Natural History, 2559 Puesta del Sol Road,
Santa Barbara, California 93105-2936, U.S.A., insignis_one@yahoo.com

Abstract: The hairy chiton *Mopalia kennerleyi* Carpenter, 1864 is distinguished from its congener *Mopalia ciliata* (Sowerby, 1840), and the identity of *Chiton wosnessenskii* von Middendorff, 1847 is clarified. *Mopalia kennerleyi* and *M. ciliata* are distinguished by setae structure, valve sculpture, and radular teeth. Their characteristics are illustrated and discussed, and their distributions defined.

Key words: chiton, sibling species, California, Polyplacophora, mollusc

The examination of several hundred lots of what has been regarded as *Mopalia ciliata* (Sowerby, 1840) from throughout its recorded range of Alaska to Baja California revealed that two similar but distinctive species could be distinguished by setae structure: *Mopalia ciliata* from southern California and Baja, and a northern species ranging from central California, north to the Aleutian Islands in Alaska, for which *Mopalia kennerleyi* Carpenter, 1864 appears to be the oldest available name. These distinctions in setae and name for the northern species have already been noted and illustrated by Eernisse *et al.* (2007). Recent molecular work by Kelly *et al.* (2007) and Kelly and Eernisse (2007) have clearly verified this conclusion.

It is not new to consider northern specimens as distinct. Pilsbry (1892: 305) distinguished *Mopalia ciliata,* from what he considered its variety *M. c. wosnessenskii* (von Middendorff, 1847), by the "much fainter sculpture" and by the latter's lack of "white thorns or spines (spicules) near the base of the setae." As Middendorff's name is currently regarded as a synonym of *M. ciliata*, one would first expect that this name should be revived for the northern taxon. However, an examination of the lectotype (Fig. 1; ZISP N834) (designated by Sirenko, pers. comm., October 2007), the larger of two syntypes of Middendorff's *Chiton wosnessenskii* (Fig. 1) revealed that it was instead a specimen of *Mopalia hindsii* (Sowerby MS, Reeve, 1847). The type locality for *C. wosnessenskii* is given as Atka Island in the Aleutians (52°11′57″N, 174°12′48″W); however, *M. hindsii* does not occur in the Aleutians. Middendorff's type specimens were said to have come from both Atka Island and Sitka, Baranof Island, SE Alaska (57°08′N, 135°55′W), the Russian capitol of Alaska during the early 1800s and the type locality of many of Middendorff's types. Undoubtedly both specimens came from Sitka. The western-most distribution of *M. hindsii* is in the vicinity of Kodiak Island, in the Gulf of Alaska (57°N, 154°W). The question of name priority for these two nominal taxa is a matter for further investigation.

Pilsbry (1892) considered *Mopalia kennerleyi* Carpenter, 1864 to be a synonym of *Mopalia ciliata*, an assignment followed by Burghardt and Burghardt (1969), Smith (1977), and Kaas and Van Belle (1994).

Although the type of *Mopalia kennerleyi* is lost (Smith 1977, T. Nickens, USNM, pers. comm. October 2003), there can be little doubt from Carpenter's original description and the type locality of "Puget Sound" as to which species he was referring. *Mopalia kennerleyi* Carpenter, 1864 is thereby reinstated as the oldest available name for northern species.

MATERIALS AND METHODS

Specimens of "*Mopalia ciliata*" from my own collection, comprising more than one hundred lots from the Aleutian Islands to Baja California, were separated into two distinctive types of girdle setae using a dissecting microscope (as in Clark 1991). Setae and radula from each of these two potential species were prepared and examined with a scanning electron microscope (SEM) at the Biology Department of Southern Oregon University, using methods described by

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[1] Mailing Address: 3808 E. Pinehurst Drive, Eagle Mountain, Utah 84005-6007, U.S.A.

* From the symposium "Advances in Chiton Research" presented at the joint meeting of the American Malacological Society and Western Society of Malacologists, held 29 July to 3 August 2006 in Seattle, Washington.

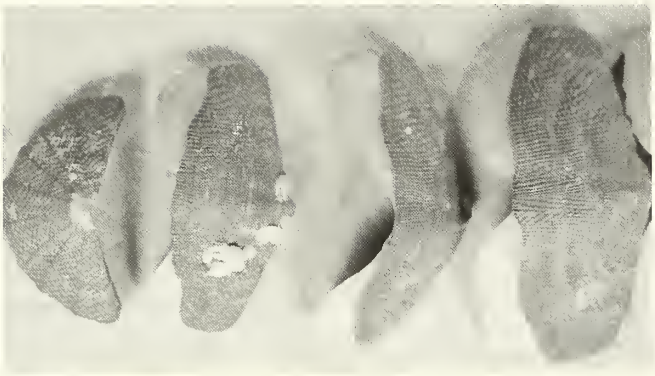

Figure 1. *Chiton wosnessenskii* von Middendorff, 1847. Lectotype, ZISP N834.

Clark (1994). The extensive collections at the Los Angeles County Museum of Natural History, California Academy of Sciences, San Diego Natural History Museum, Santa Barbara Museum of Natural History, and the Royal British Columbia Museum were also examined. The higher level systematics used here follows Sirenko (2006).

Acronyms used in the text are as follows: ZISP, Zoological Institute, Academy of Sciences, Saint Petersburg; LACM, Los Angeles County Museum of Natural History; RNC, Roger N. Clark, personal reference collection.

SYSTEMATICS

Class: Polyplacophora Gray, 1821
Order: Chitonida Thiele, 1910
Suborder: Acanthochitonina Bergenhayn, 1930
Family: Mopaliidae Dall, 1889
Genus: *Mopalia* Gray, 1847
Type Species: *Chiton hindsii* Sowerby, MS, Reeve, 1847, by subsequent designation.

Mopalia ciliata (Sowerby, 1840)
Mopalia kennerleyi Carpenter, 1864

Mopalia kennerleyi Carpenter, 1864
(Figs. 2-6)

Mopalia kennerleyi Carpenter, 1864: 648; Eernisse *et al.* 2007; Kelly and Eernisse 2007; Kelly *et al.* 2007.
Mopalia grayii Carpenter, 1864: 603 (*nom. nud.*).
Chaetopleura thouarsiana de Rochebrune, 1882: 191.
Mopalia ciliata var. *wosnessenskii* (Middendorff): Pilsbry, 1892: 305; Leloup 1942: 49.
Mopalia muscosa kennerleyi Carpenter, 1864; Dall 1921: 195; Oldroyd 1924: 197; Oldroyd 1927: 306.
Mopalia ciliata wosnessenskii (Middendorff): Abbott 1974: 305.
Mopalia ciliata (Sowerby): Burghardt and Burghardt 1969: 26 (in part); Smith 1977: 250 (in part); Putman 1980: 122 (in part); Baxter 1987: 105; Kaas and Van Belle 1994: 222 (in part).

Diagnosis

Medium sized (to 6.5 cm), oval chitons; valves subcarinated to rounded, depressed to moderately elevated, weakly sculptured. Girdle with strap-like or trough-shaped setae, bearing two rows of pointed spicules to about 250 μm in length. Valves variously patterned with green, black, brown, yellow, red, and white.

Description

Chitons of medium size, neotype designated herein (Fig. 2) 49 × 27.5 mm; shell oval, subcarinated to rounded, low to

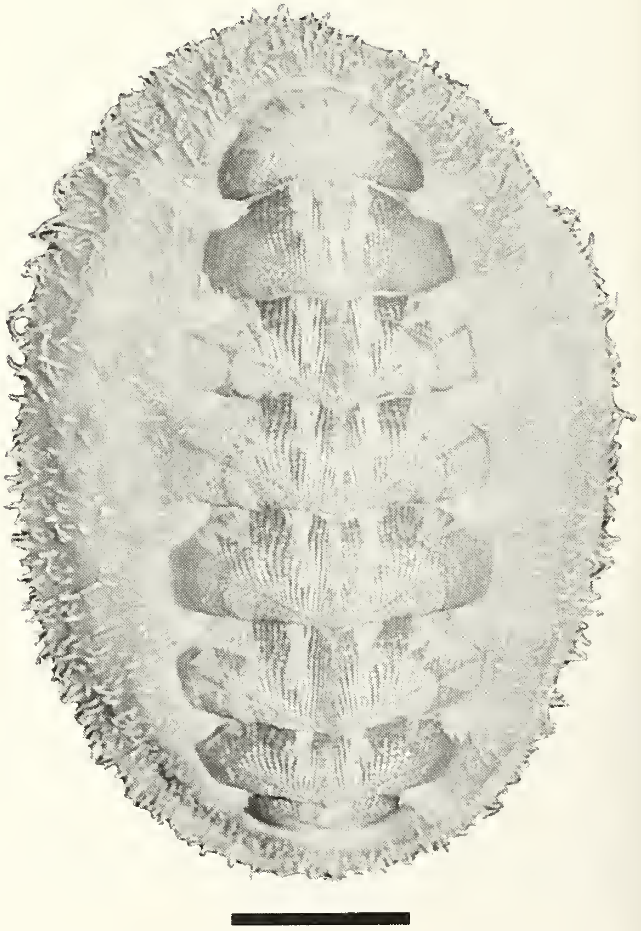

Figure 2. *Mopalia kennerleyi* Carpenter, 1864. Neotype, LACM 2886. Scale bar = 10 mm.

moderately elevated, beaked. Head valve and lateral areas of intermediate valves defined with rows of low, weak, flattened pustules; interstices of head valve and surface of lateral areas with radial rows of low, broad pustules, often coalescing into irregular zigzaging costae; central areas with smooth to granular, longitudinal (gently, posteriorly curved) ribs, or rows of low, coalescing, oval pustules. Tail valve small, oval, about twice as wide or more than long, with distinctive wide posterior sinus.

Girdle wide, usually more than one half the width of intermediate valves, notched posteriorly; dorsal surface bearing short (2-3 mm), slender trough-like or flattened (strap-like) setae (Figs. 3-5) with (normally) two rows of slender, curved, sharp spicules to about 250 μm in length (Figs. 4-5); ventral surface of girdle covered with minute, broad, flattened, distally pointed spicules to about 125 × 34 μm; margin of girdle with similar, but longer spicules, to about 180 μm.

Radula (Fig. 6), typical of a member of Mopaliidae, with large, robust major lateral teeth, bearing tridentate denticle caps; central cusp the longest, inner cusp slightly shorter, and outer cusp only about one third as long as the central; central tooth subquaudrate, tapering proximally, and indented slightly just below cutting edge.

Ctenidia merobranchial, abanal, extending about 80% of foot length, from beneath valve two to the suture of valves seven and eight; about 42 per side in animals 50 mm in length.

Color: Valves variously patterned (Figs. 7-12) with green, brown, yellow, black, red, and white, often variegated, mottled, or suffused. Entire valves or portions one or more valves often unicolored. Girdle yellowish or brown.

Type material

Type lost (Smith 1977); NEOTYPE, LACM 2886 (*leg.* S. R. Thorpe, 1 July 1965) (Fig. 2).

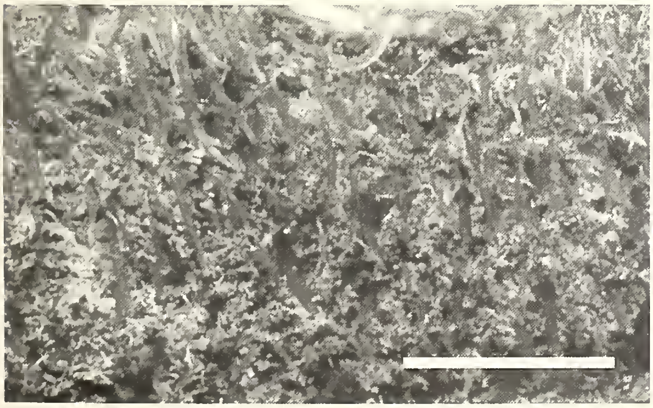

Figure 3. *Mopalia kennerleyi.* Close-up of girdle. Scale bar = 3 mm.

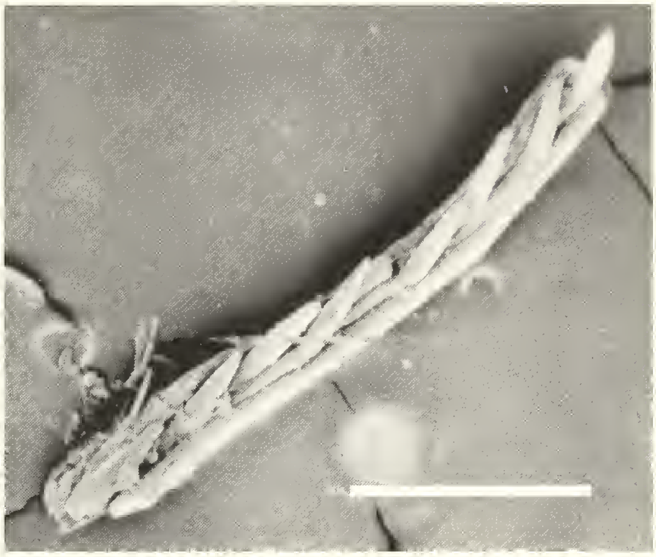

Figure 4. *Mopalia kennerleyi.* SEM image of seta. Scale bar = 250 μm.

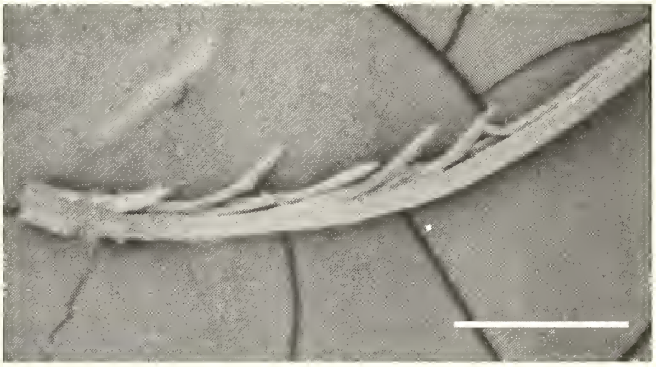

Figure 5. *Mopalia kennerleyi.* SEM image of seta. Scale bar = 250 μm.

Type locality

"Puget Sound", herein restricted to Tacoma Narrows, Pierce County, Washington, U.S.A. (47°16′N, 122°31′W), intertidal.

Distribution

Mopalia kennerleyi is a North American boreal species, occurring from the southern Bering Sea (to about 54°N) and Aleutian Islands (west to Attu Island, 173°14′E; LACM 79-72) and Gulf of Alaska (north to 61°N) south to Monterey Bay, California (36°36′N; RNC 1988), but rare south of San Mateo County, California (37°10′N), where it begins to be replaced by the similar *Mopalia ciliata.* In Monterey Bay it is always subtidal, at about 10-15 m.

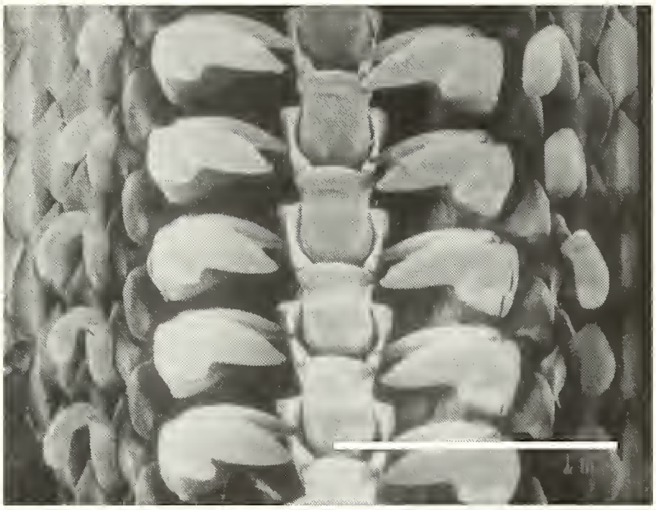

Figure 6. *Mopalia kennerleyi.* SEM image of radula. Scale bar = 500 µm.

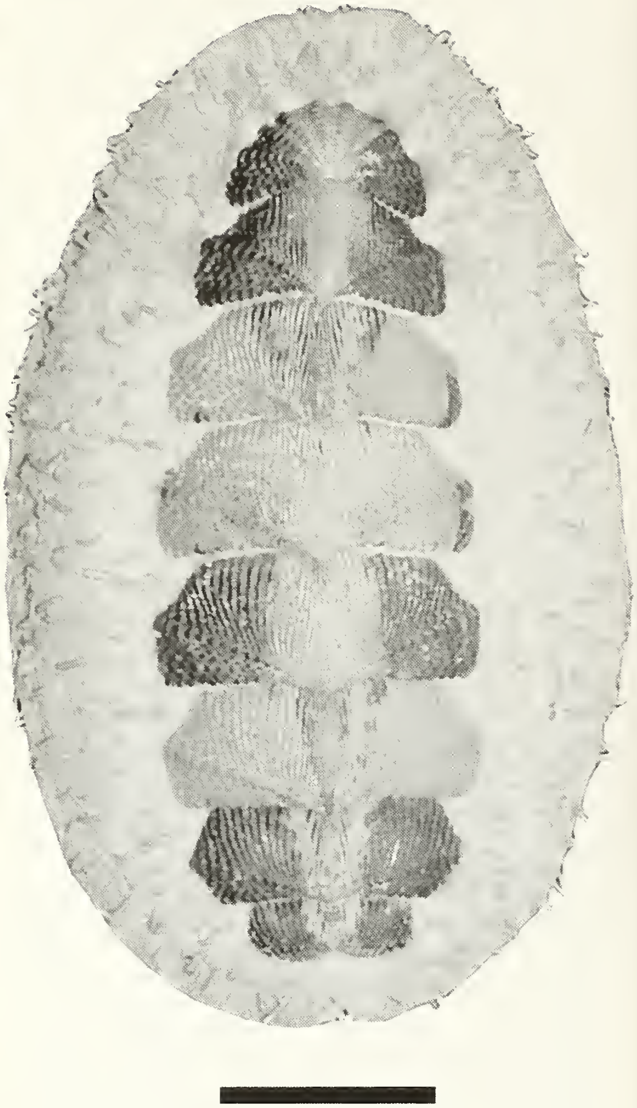

Figure 7. *Mopalia ciliata* (Sowerby, 1840). Neotype, LACM 2885.

Mopalia ciliata (Sowerby, 1840)
(Figs. 7-10)

Chiton ciliata Sowerby, 1840: 289

Mopalia ciliata (Sowerby): Pilsbry 1892: 303; Leloup 1942: 49; Burghardt and Burghardt 1969: 26 (in part); Abbott 1974: 401; Putman 1980: 122 (in part); Clark 1991: 312; Kaas and Van Belle 1994: 222 (in part); Eernisse *et al.* 2007; Kelly and Eernisse 2007; Kelly *et al.* 2007.

Diagnosis

Medium sized (to 5.0 cm), oval to elongate-oval chiton; valves carinated, moderately elevated, finely sculptured. Girdle with short, strap-like setae bearing (normally) four rows of large, white, pointed spicules. Color variable, often suffused or mottled with pale green, white or dark brown, sometimes olive, with white, orange, red, and blue markings.

Description

Chitons of medium size, neotype 46 × 28 × 8 mm (Fig. 7). Shell elongate-oval, valves carinated, moderately elevated, beaked. Head valve and lateral areas of intermediate valves defined with radial rows of fairly heavy, round or irregular pustules; interstices of head valve and surface of lateral areas with large, low, round or irregular pustules, which may be spaced or touching; posterior edge of lateral areas dentated by oval pustules; central areas with longitudinal rows of low, oval pustules or smooth ribs. Girdle moderately wide, about 1/2 to 3/4 the width of valves, notched posteriorly; dorsal surface spiculose, spicules to about 250 × 25 µm, occurring (mostly) singularly, or in groups of two to four, as well as short (2-3 mm), strap-like setae (Figs. 8-9) bearing four rows or robust, white spicules, to 600 µm in length (often present only on lower half of seta); ventral surface of girdle with scattered spicules to 150 × 32 µm; margin of girdle with similar but longer spicules to about 250 µm. Radula (Fig. 10), with robust major lateral teeth bearing tridentate denticle caps; central cusp longest, inner one slightly shorter, and outer cusp about 1/3 as long as the inner one. Ctenidia, merobranchial, abanal, extending about 80% of foot length, from about the suture of valves two and three, to the suture of valves seven and eight, 34 per side in neotype.

Color: Variable, neotype green with black-brown markings. This according to Pilsbry (1892) is the "typical" coloration of the species, based presumably on Pilsbry's exami-

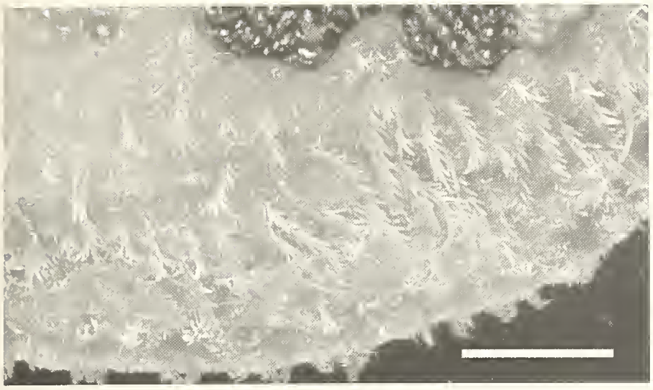

Figure 8. *Mopalia ciliata.* Close-up of girdle. Scale bar = 3 mm.

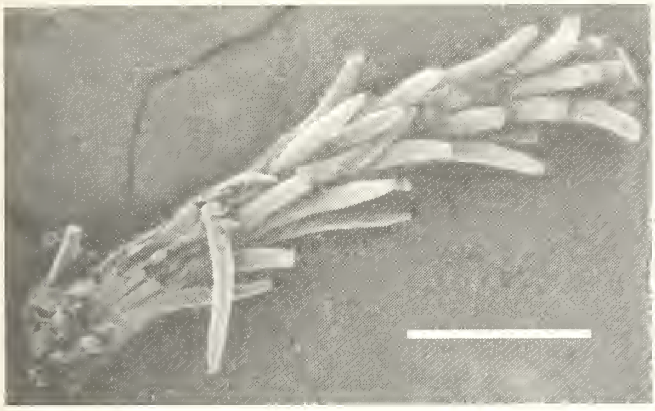

Figure 9. *Mopalia ciliata.* SEM image of setae. Scale bar = 500 μm.

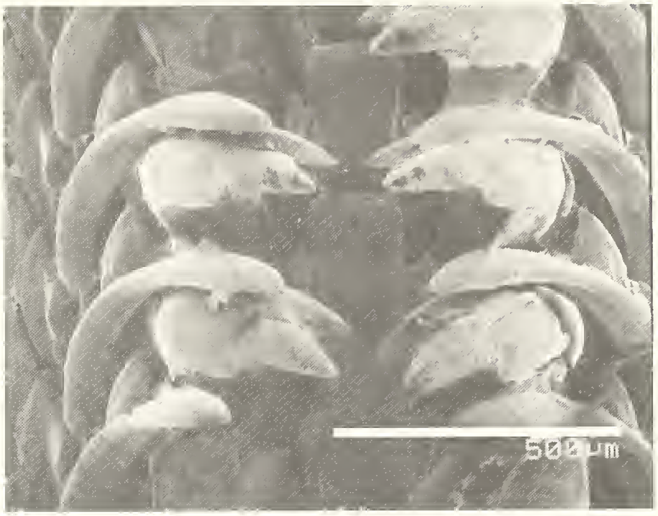

Figure 10. *Mopalia ciliata.* SEM image of radula. Scale bar = 500 μm.

nation of Sowerby's material or original description. From the material examined, this is the typical color morph of southern California (Los Angeles and Orange Counties) populations.

Type material

Type, BMNH? Not located (K. Way, *in lit.*, 26 July 2000). Presumed lost. NEOTYPE, LACM 2885 (*leg.* Spencer R. Thorpe, Jr., low intertidal on rock, 11 December 1958).

Type locality

White Point, Los Angeles County, California, U.S.A. (33°42.8′N, 118°08.3′W).

Distribution

Mopalia ciliata is a North American, warm-temperate species, occurring between latitudes 38°40′N (RNC 1308), Sonoma County, California and 30°20′N (LACM 66-3), Rancho Soccoro, Baja California. The species is rare north of San Mateo County, California (37°10′N), where it is mostly replaced by *Mopalia kennerleyi.*

DISCUSSION

Although Pilsbry (1892) separated these two taxa as subspecies (using von Middendorff's *Chiton wosnessenskii* for the taxon presently regarded as *Mopalia kennerleyi*) based on the large prominent, white spicules present on the setae of *M. ciliata* and absent in "*M. wosnessenskii*," recent workers have had much difficulty separating the two.

The similarities between these two species are remarkable, as they mimic each other in the form and sculpture of the plates, as well as their coloration. Hybrids are unknown in *Mopalia*; none of the other eighteen Pacific coast species exhibit this tendency. However, in central California, a few specimens have been found that indicate further investigation into the possibility of hybridization might be warranted.

Mopalia ciliata is particularly puzzling, exhibiting a myriad of forms, some with broad plates, some rather narrow, and the sculpture varying from delicate and nearly smooth to quite coarse. Additionally, many animals in the Monterey Bay area often resemble *Mopalia spectabilis* Cowan and Cowan, 1977, with greenish (olive to turquoise) plates marked with brilliant blue zigzag lines and red flecks, but they are easily distinguished from that species by the generally broader outline, and the structure of the setae.

The most diagnostic character for distinguishing *Mopalia ciliata* and *Mopalia kennerleyi* (as well as all other species of *Mopalia*) is the structure of the setae. A comparison of the setae (best taken from the central portion of the girdle) reveals that the setae of *Mopalia ciliata* are flatter, broader, and bear (when fully mature) four rows of large, robust, sharp, curved, white calcareous spicules, to about 600 µm in length. The setae of *M. kennerleyi* are more slender, trough-shaped (often nearly tubular) and bear (when fully mature) two rows of slender, slightly curved, sharp spicules, similar to those in *M. ciliata*, but much smaller, reaching only about 250 µm in length. Another useful distinction is that the posterior valve margin has denticulations in *M. ciliata*; these are lacking in *M. kennerleyi.*

The radulae of the two species are very similar, the teeth are nearly identical in shape and proportion, but those of *Mopalia kennerleyi* are proportionally about 20% larger than those of *Mopalia ciliata* of the same size. Also the middle cusp of the denticle cap of the major lateral teeth is slightly longer in *M. kennerleyi.*

Taken together, these characters along with their geographic separation provide ample evidence that *Mopalia ciliata* and *Mopalia kennerleyi* are both valid species.

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Two new chitons of the genus *Tripoplax* Berry, 1919 from the Monterey Sea Canyon*

Roger N. Clark[1]

Department of Invertebrate Zoology, Santa Barbara Museum of Natural History, 2559 Puesta del Sol Road,
Santa Barbara, California 93105-2936, U.S.A., insignis_one@yahoo.com

Abstract: Recent deep-sea trawling in the Monterey Sea Canyon, California has brought to light two previously unknown bathyal chitons. The new species, members of the family Ischnochitonidae, are placed in the genus *Tripoplax* Berry, 1919, here in raised to full generic rank on the basis of morphological and ecological characteristics. *Tripoplax calypso* spec. nov. and *Tripoplax cowani* spec. nov. are described, illustrated, and compared to similar species from the region.

Key words: Polyplacophora, new species, mollusc, Monterey Bay

The Monterey submarine canyon is a gigantic undersea chasm, starting just offshore in less than 10 m of water, and plunging to depths of more than 3000 m just 50 km offshore. The Carmel Canyon is the southern branch of this system. The fauna of these canyons is extremely rich and diverse, and is being intensely studied by the Monterey Bay Aquarium Research Institute and the Moss Landing Marine Laboratories.

Deep-sea trawling in the canyons by the research vessels USNS *DE Steiguer* (1975) and the R/V *Point Sur* (1994-1998) have procured several specimens of two undescribed chitons of the genus *Tripoplax* Berry, 1919. *Tripoplax calypso* spec. nov. and *Tripoplax cowani* spec. nov. were taken at depths of 650-1044 m on rocks and sponges. The new species are compared to the similar *Lepidozona retiporosa* (Carpenter, 1864), *Lepidozona scrobiculata* (von Middendorff, 1847) and *Lepidozona golischi* (Berry, 1919), and *Tripoplax abyssicola* (Smith and Cowan, 1966), and *Stenosemus stearnsii* (Dall, 1902), respectively.

Both new species are members of the genus *Tripoplax* Berry, 1919, herein elevated to full generic status and characterized by fine tegmental sculpturing, relatively small girdle scales (~300 μm), and multiple slits in the insertion plates of the intermediate valves, in contrast to the genus *Lepidozona* Pilsbry, 1892 which has single-slitted intermediate valves and usually coarser (often pustulose or tuberculose) sculpturing. Additionally, all members of *Tripoplax* are cold northern or deep water inhabitants, distributed in the northern Pacific Ocean between latitudes 36°N (off central California, U.S.A.) and 37°N (northern Honshu, Japan) and 60°N in the Gulf of Alaska and Okhotsk Sea, in cool temperate to sub-arctic and bathy-abyssal waters, restricted to temperatures below about 9 °C. *Tripoplax* reaches its greatest diversity in the Aleutian Islands of Alaska, where seven species are presently known. Members of *Lepidozona* are distributed nearly worldwide, and they typically inhabit warmer, temperate to tropical waters, at depths of 400 m or less. Most inhabit shallow (1-50 m) subtidal waters. Only four species of *Lepidozona* are found in the Gulf of Alaska north of 50°N: *Lepidozona mertensii* (von Middendorff, 1847), *Lepidozona willetti* (Berry, 1917), *Lepidozona retiporosa*, and *Lepidozona golischi*. The genus is absent in the Aleutian Islands, and only the species *Lepidozona multiganosa* Sirenko, 1978 is found in the southern Okhotsk Sea, near the southern Kurile Islands, north to Urup Island (46°N). No species of *Lepidozona* occur in the north Pacific region between 152°W, east of Kodiak Island, Alaska and about 150°E, east of Urup Island, Kurile Islands, Russia.

Berry (1919) briefly defined *Tripoplax* for the Alaskan species *Ischnochiton* (*Trachydermon*) *trifidus* Carpenter, 1864, a species that Dall (1871) had erroneously placed in the subgenus *Ischnoradsia* Shuttleworth, 1853 with *Chiton australis* Sowerby, 1840. Realizing that these two taxa were only distantly related, Berry separated the two species because of the rather smooth tegmental sculpturing and relatively small girdle scales (to 315 × 250 μm) possessed by *I. trifidus*, in contrast with the coarse sculpture and very large (to 600 μm in width), subcarinated girdle scales of *I. australis. Ischnoradsia* is presently regarded as monotypic subgenus for *Ischnochiton australis* (Sowerby) by Kaas and Van Belle (1990). The validity of *Trachydermon* is somewhat unclear, at the time of its very brief description and subsequent usage by Carpenter; it was an assemblage of several unrelated species, presently placed in three different families, without an original type designation. Kaas and Van Belle (1985) considered it a synonym of *Lepidochitona* Gray, 1821, but that may be invalid as well, and a re-evaluation of this name, its validity, and position is clearly necessary (see Palmer 1958: 284). Kaas and Van Belle (1987) used *Tripoplax* as a subgenus of *Lepidozona* Pilsbry, 1892 for members of that genus with multiple slits in the insertion plates of the intermediate valves. This combination was followed by Clark (1991, 2000).

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[1] Mailing Address: 3808 E. Pinehurst Drive, Eagle Mountain, Utah 84005-6007, U.S.A.

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In its morphology and biogeography, *Tripoplax* appears to be a natural assemblage. Molecular studies would be valuable to test this hypothesis and its relationship to *Lepidozona*.

Acronyms used in the text are: CASIZ, California Academy of Sciences, Invertebrate Zoology; LACM, Los Angeles County Museum of Natural History; ZIAS, Zoological Institute, Academy of Sciences, Saint Petersburg, Russia; RNC, Roger N. Clark personal collection.

SYSTEMATICS

Class: POLYPLACOPHORA Gray, 1821
Order: Chitonida Thiele, 1909
Family: Ischnochitonidae Dall, 1889
Genus: *Tripoplax* Berry, 1919

Type species: *Ischnochiton* (*Trachydermon*) *trifidus* Carpenter, 1864, by original designation
Ischnoradsia Carpenter MS, Dall, 1871, non Shuttleworth, 1853; *Gurjanovillia* Jakovleva, 1952; *Albrechtia* I. Taki, 1955

Expanded definition

Small to medium sized chitons (1-6.5 cm), elongate-oval to broadly oval in outline; central areas with fine pitting, net-like reticulation of cross threading or finely beaded longitudinal lirae, crossed by growth lines or very fine transverse lirae. Radial areas with relatively weak or very fine sculpturing; insertion plates of intermediate valves with two to four slits. Dorsal girdle scales relatively small (200-320 μm in length), smooth, or bearing minute riblets or striations, and often mammillated at apices.

Tripoplax calypso spec. nov.
Tripoplax cowani spec. nov.

Tripoplax calypso spec. nov.
(Figs. 1-7)

Diagnosis

Small (to 17 mm), oval chitons; valves carinated, slopes convex; lateral areas with three to five low, faint radial ribs separated by weak sulci; central areas with 26-28 curved, longitudinal riblets; girdle with imbicating, striated scales to 200 × 160 μm; radula with heavy, bidentate major lateral

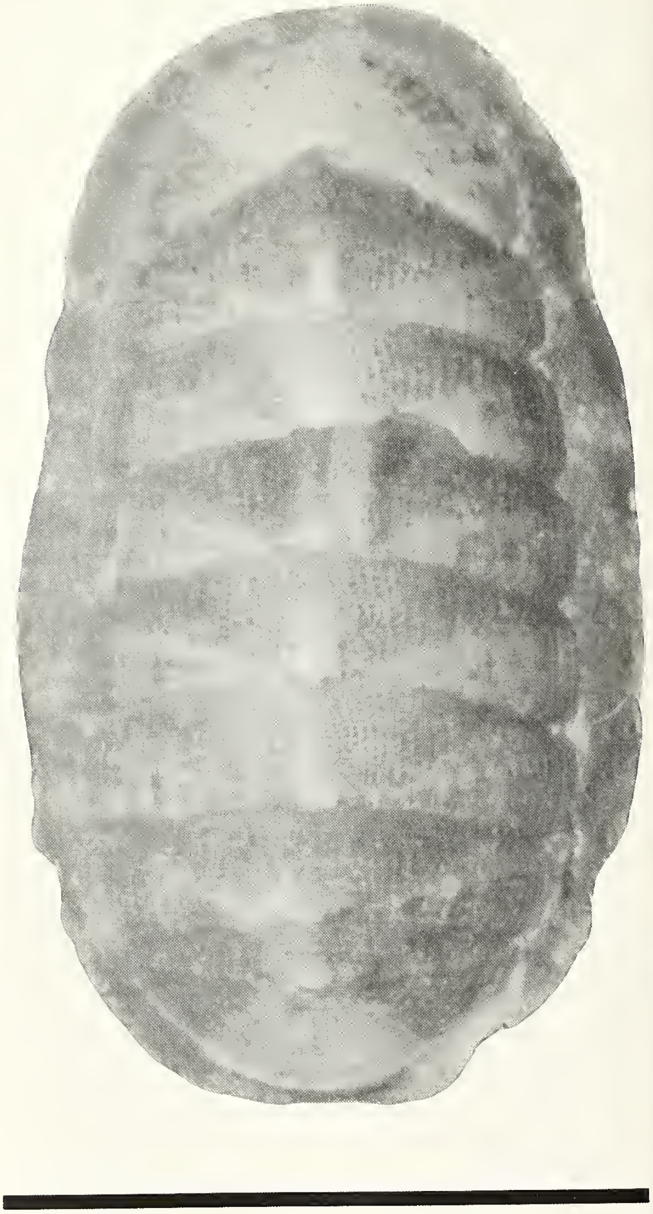

Figure 1. *Tripoplax calypso* Clark, spec. nov. Paratype (RNC 2060), scale bar = 10 mm.

teeth. Color: valves and girdle dull reddish-brown with some white patches.

Description

Holotype (Figs. 2-7) small (13.5 × 8.2 × 2.7 mm), oval, moderately elevated; valves granular, carinated, side slopes convex, tegmentum delicately sculptured. Head valve (Fig. 2) semi-circular, posterior margin widely V-shaped, bearing 23 low, faint (nearly obsolete), rounded radial ribs. Intermediate valves (Figs. 3-4) oblong, about four times as wide as long; lateral areas with three to five low, faint radial ribs, separated by faint sulci; central areas with about fourteen curved, longitudinal ribs per side, becoming obsolete at the jugum; the ribs (when viewed dorsally) are seen to be made up of faint transverse riblets with numerous raised posterior extensions, which overlap the next rib in the series, as if made up of overlapping drips; jugal areas show only obsolete pitting. Tail valve (Fig. 5) relatively large, almost diamond shaped; mucro ante-central, slightly raised; postmucronal slope concave; ante-mucronal area with obsolete pitting; terminal area with about 21 low, faint radial ribs. Articulamentum white, insertion teeth short, blunt; sutural laminae short, round, connected across the jugal sinus by a short, concave jugal plate with slits at edges; slit formula 11/2/13. Girdle narrow, about one fifth as wide as intermediate valve five; clothed dorsally with imbricating, oval, striated scales (Fig. 6) to about 200 × 160 μm, and bearing 15-16 rather weak riblets; margin of girdle with minute, pointed spicules to 60 × 18 μm; ventral surface of girdle with radiating rows of minute, rectangular scales to 100 × 15 μm. Radula (Fig. 7) 6.0 mm long, bearing 40 mature rows of teeth; rachidean tooth hourglass shaped, working edge about 50 μm wide; minor lateral teeth wing-shaped, with small, lateral extension near the anterolateral edge; major laterals relatively large, with bidentate denticle cap, the inner cusp about twice as long as outer cusp. Ctenidia holobranchial, adanal about 17 per side. Color: dull reddish-brown with white patches on terminal valves, and jugal areas of some intermediate valves. Paratype (Fig. 1) agrees with holotype in all aspects, but is larger (17.0 mm × 10.0 mm × 3.1 mm), and has 18 ctenidia per side.

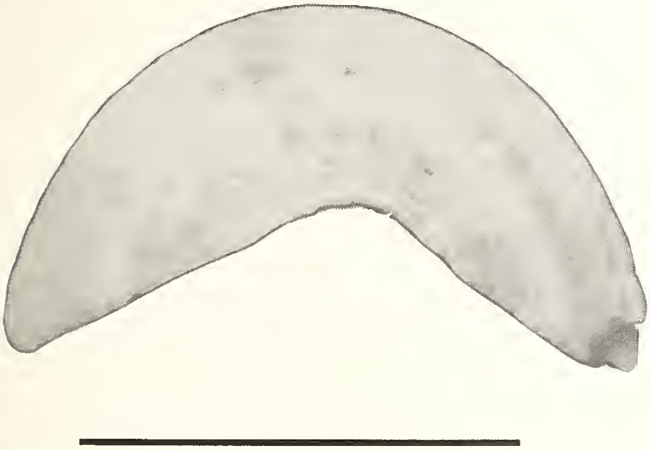

Figure 2. *Tripoplax calypso* Clark, spec. nov. Holotype (LACM 2882). Head valve, scale bar = 5 mm.

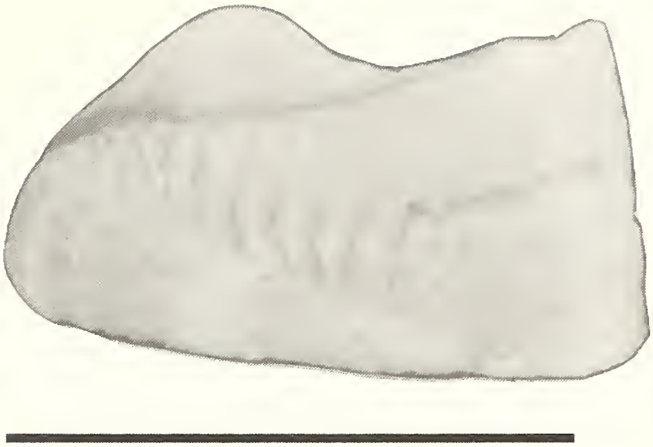

Figure 3. *Tripoplax calypso* Clark, spec. nov. Holotype (LACM 2882). Intermediate valve fragments, scale bar = 5 mm.

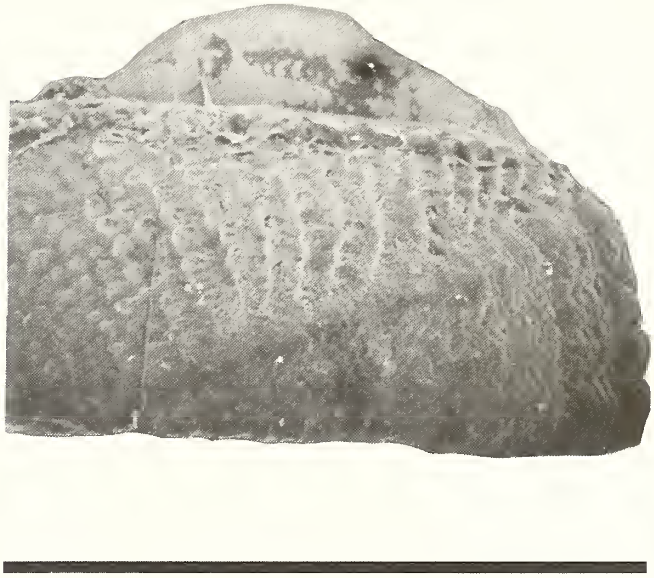

Figure 4. *Tripoplax calypso* Clark, spec. nov. Holotype (LACM 2882). Intermediate valve fragments, scale bar = 5 mm. SEM image.

Type material: Holotype (LACM 2882); radula, girdle, and intermediate valve fragment mounted on SEM

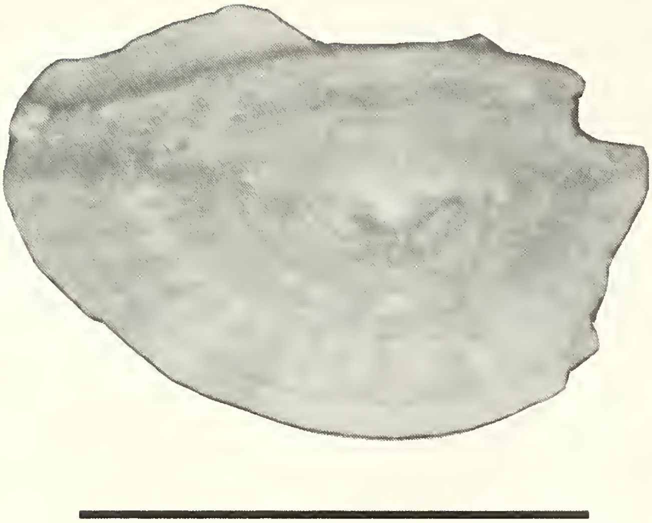

Figure 5. *Tripoplax calypso* Clark, spec. nov. Holotype (LACM 2882). Tail valve, scale bar = 5 mm.

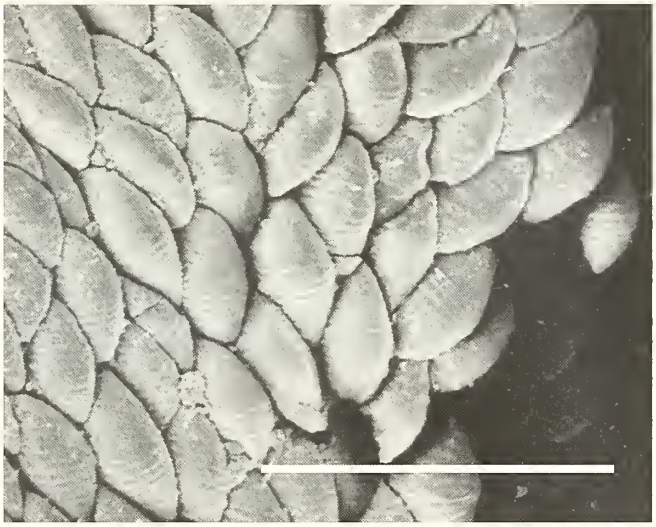

Figure 6. *Tripoplax calypso* Clark, spec. nov. Holotype (LACM 2882). Dorsal girdle scales, scale bar = 500 µm.

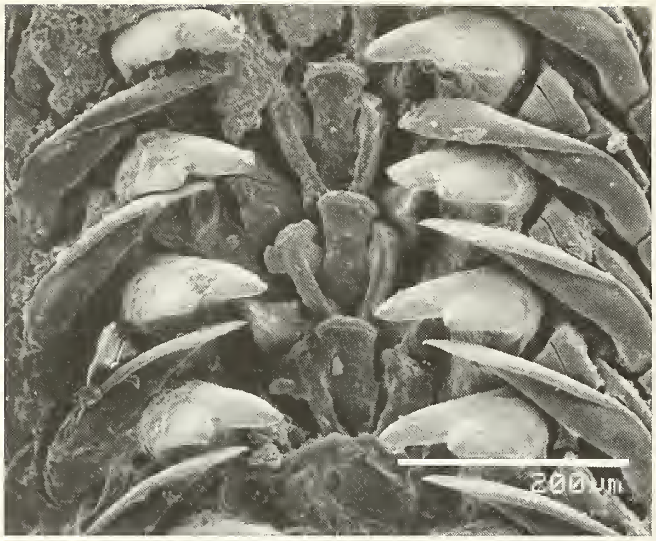

Figure 7. *Tripoplax calypso* Clark, spec. nov. Holotype (LACM 2882). Radula, scale bar = 200 µm.

viewing stub; head and tail valves, and intermediate valve fragments separate, unmounted; body in ethanol (*leg.* Roger N. Clark, 28 September 1995; trawled R/V *Point Sur*).

Paratype (RNC 2060), whole animal in ethanol (*leg.* Roger N. Clark, 6 March 1997; trawled, R/V *Point Sur*).

Type locality: California, Monterey County, Monterey Submarine Canyon (36°45.163′N, 122°03.447′W), 650-700 m.

Habitat and ecology: The holotypes and paratypes were found living on large dead chunks of the massive, ridged, hexactinellid sponges *Aphrocallistes vastus* and *Heterochone calyx.* Another chiton, *Stenosemus stearnsii* was also found in this habitat.

Four additional chitons were found on rocks in the same trawls: *Tripoplax cowani* new species, *Placiphorella pacifica* Berry, 1919, *Leptochiton mesogonus* (Dall 1902), and *Leptochiton* sp.

Etymology: The name comes from Greek mythology, Calypso, the nymph who hid Ulysses.

Remarks: At first sight *Tripoplax calypso* merely looks like a deep-water specimen of *Lepidozona retiporosa* (Fig. 8) it is only when examined under magnification that the unique sculpture of the valves becomes evident. Still it might be passed off as a form of the latter, or perhaps considered to be within the considerable range of variation attributed to *Lepidozona scrobiculata* [as *Lepidozona sinudentata* (Carpenter in Pilsbry, 1892), Ferreira 1978, Kaas and Van Belle 1987] (Fig. 9). Although the multiple slits in the intermediate valves (lacking in both of the previous species) and the very faint radial sculpture serve to distinguish it. Additionally, the girdle scales of *T. calypso* reach about 200 × 160 µm and have 15-16 weak riblets, while those of *L. retiporosa* reach only about 145 × 120 µm and have eight to ten riblets, and the scales of *L. scrobiculata* reach 185 × 130 µm and have 10-13 weak riblets. *Lepidozona golischi* (Berry, 1919) [*Lepidozona scabricostata* (Carpenter) of Ferreira 1978, Kaas and Van

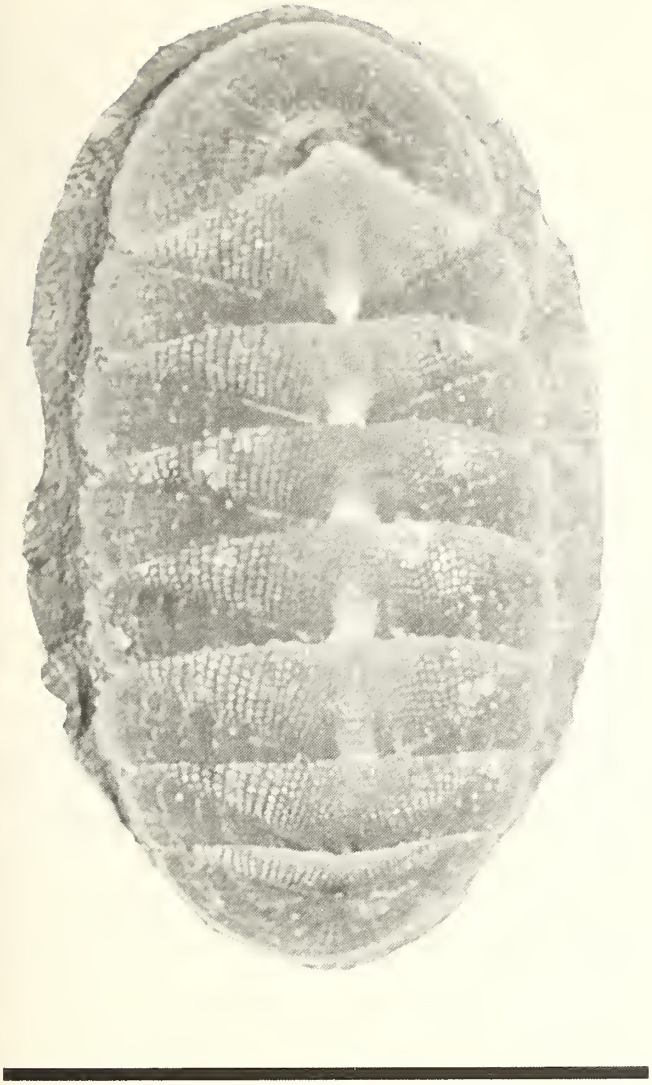

Figure 8. *Lepidozona retiporosa* (Carpenter 1864). Scale bar = 10 mm. Eernisse coll., San Juan Island, Washington, depth unknown.

Belle 1987, and Clark 1991, non *L. scabricostata* (Carpenter, 1864)] (Fig. 10) might also be confused with this species but is generally uniformly pale orange, tan, or white in color; central areas are ribbed, radial areas are rather flat, sometimes with sulci, and bear relatively large, often scattered pustules and differently proportioned girdle scales reaching 130 × 220 µm and bearing 15-16 riblets.

Tripoplax cowani spec. nov.
(Figs. 11-14)

Ischnochiton abyssicola Smith and Cowan, 1966 (in part)

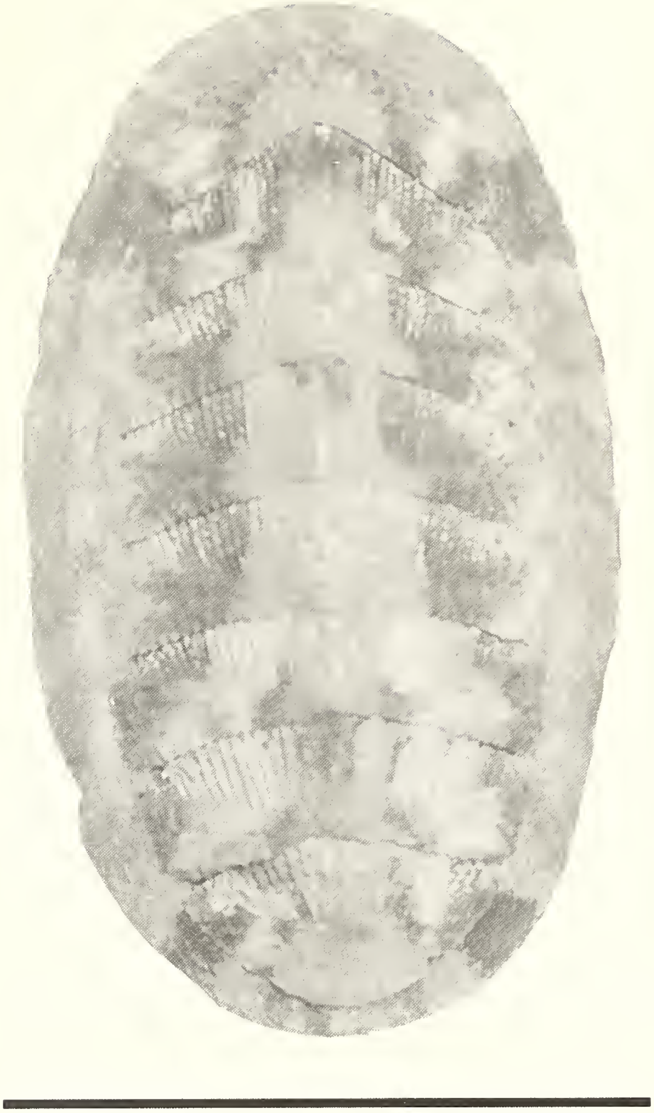

Figure 9. *Lepidozona scrobiculata* (von Middendorff, 1847). (RNC 244). Monterey Bay, California, 15 m, scale bar = 10 mm.

Diagnosis

Medium sized (to 4.5 cm) oval chitons; valves solid, carinated, moderately elevated; terminal and lateral areas with radiating rows of oval pustules; central areas with longitudinal riblets; girdle narrow, less than one sixth the width of intermediate valve five; dorsal surface clothed with small, blunt, smooth, rounded, subtriangular scales, to 300 × 275 µm. Color: white, often stained yellowish or black with deep sea mineral deposits.

Description

Holotype (Fig. 11) of medium size (38 × 22.5 × 8 mm), oval, moderately elevated; valves granular, carinated, un-

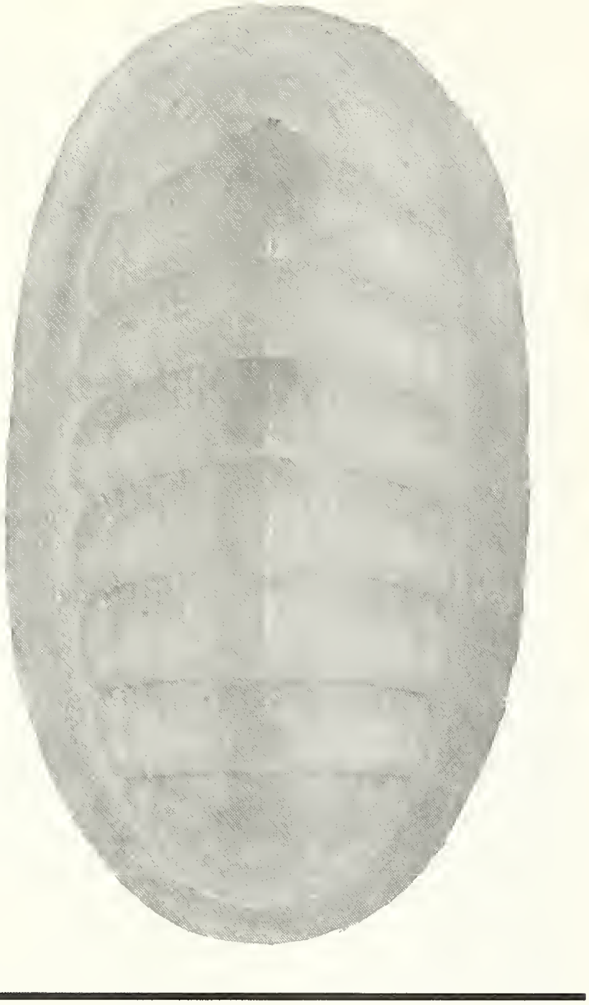

Figure 10. *Lepidozona golischi* (Berry, 1919). (RNC 616). Cape Blanco, Oregon, 34 m, scale bar = 10 mm.

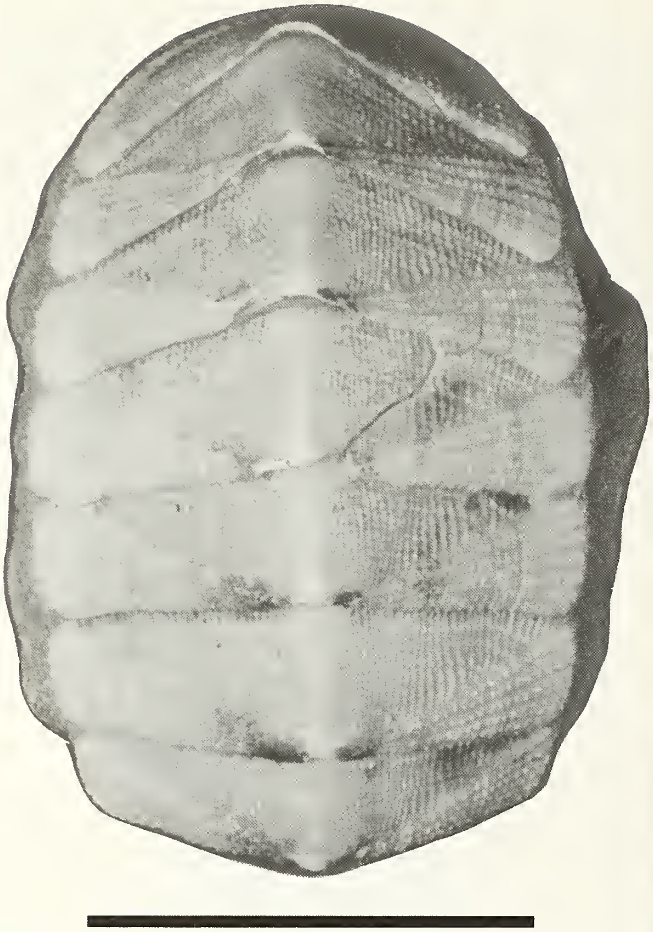

Figure 11. *Tripoplax cowani* Clark, spec. nov. Holotype (CASIZ 115832). Scale bar = 10 mm.

beaked, slopes straight to convex; tegmentum strongly sculptured. Head valve (Fig. 12) semi-circular, slope straight; posterior margin widely V-shaped, posterior edge slightly rounded; surface with 52 low, weak radiating ribs, capped with a row of oval pustules, and separated by faint sulci; intermediate valves (Fig. 13, valve V) oblong, about three times as wide as long, eaves short; lateral areas with six to eight radiating pustulose ribs, like those of the head valve; central areas with about 36 longitudinal riblets (18 per side), becoming obsolete at the jugum; jugum obsoletely pitted. Tail valve (Fig. 14) slightly convex anteriorly, rounded posteriorly, mucro ante-central, post-mucronal slope concave; ante-mucronal area with 32 longitudinal riblets (obsolete at jugum) post-mucronal area with about 43 radiating rows of pustules. Articulamnetum white, insertion teeth short, blunt; sutural laminae short, rounded connected across the moderate jugal sinus by a short, concave jugal plate with slits at edges; slit formula 16/1-3/14. Girdle (Fig. 15), narrow, about one sixth as wide as valves; dorsal surface covered with crowded, juxtaposed, smooth, blunt, rounded (subtriangular) scales to about 300 × 275 µm; margin of girdle with pointed spicules to about 250 × 20 µm; ventral surface covered with radiating rows of minute, rectangular scales 200 × 25 µm. Radula (Fig. 16) 13.5 mm long, bearing 43 mature rows of teeth; rachidean tooth about 150 µm long, broadly dilated anteriorly, working edge about 100 µm wide; minor laterals with small, anterolateral projection; major laterals large, with bidentate denticle cap, inner cusp twice as long as outer cusp. Ctenidia holobrachial, adanal, extending from beneath suture of head and second valves, to under tail valve, about 35 per side. Color: White, often stained with yellowish or black deep sea mineral deposits.

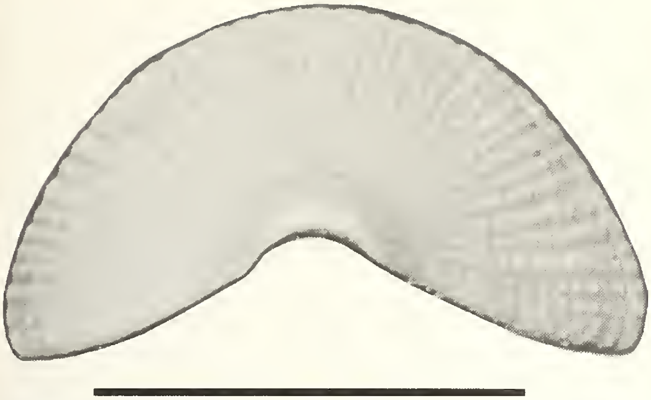

Figure 12. *Tripoplax cowani* Clark, spec. nov. Paratype (Clark 404). Head valve, scale bar = 10 mm.

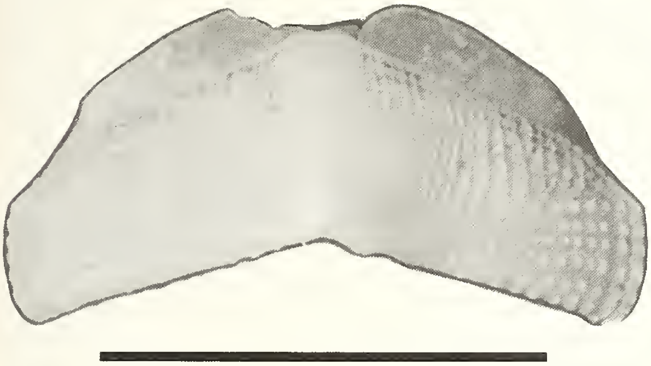

Figure 13. *Tripoplax cowani* Clark, spec. nov. Paratype (Clark 404). Intermediate valve V, scale bar = 10 mm.

Paratypes agree with the holotype in all aspects, except for variations in the number of radial ribs, and the number of slits in the articulamentum, due to the size and age of the specimens. The number of ribs on the head valves varies from 42-71, those of the lateral areas, from five to nine, and those on the tail valve from 32 to 43, slit formula range is 14-17/2-3/13-15. Paratypes range in size from 26.5 mm (LACM 2883) to ca. 45 mm (CASIZ 001640).

Type material

Holotype, CASIZ 11583, whole animal (curled) in alcohol, radula mounted on SEM stub (*leg.* USNS *DE Steiguer* 1975); Paratypes, 2, CASIZ 010602 (same data as holotype); 1, RNC 404 (same data as holotype); 1, LACM 2883; 1, ZIAS 1936; 2, RNC 2065 (*leg.* Chris Mah, 22 October 1994; trawled, R/V *Point Sur*, 650-700 m); 2, RNC 2121, west of San Francisco Bay, California (37°37.464′N, 123°05.43′W) (*leg.* R. N. Clark, 8 November 2000; trawled R/V *Miller Freeman*, 651-674 m; NMFS 21-0012-212).

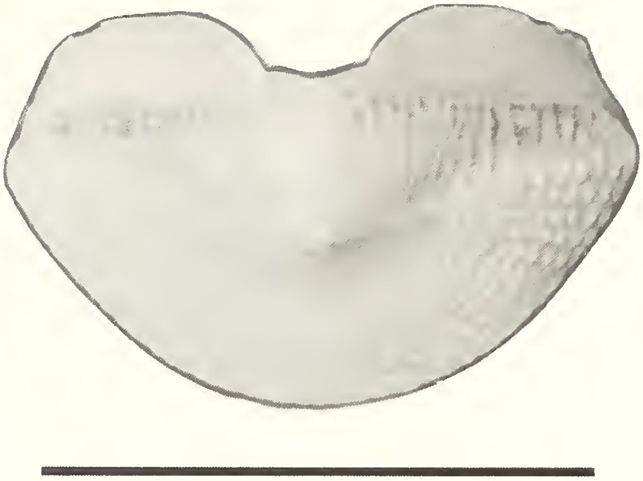

Figure 14. *Tripoplax cowani* Clark, spec. nov. Paratype (Clark 404). Tail valve, scale bar = 10 mm.

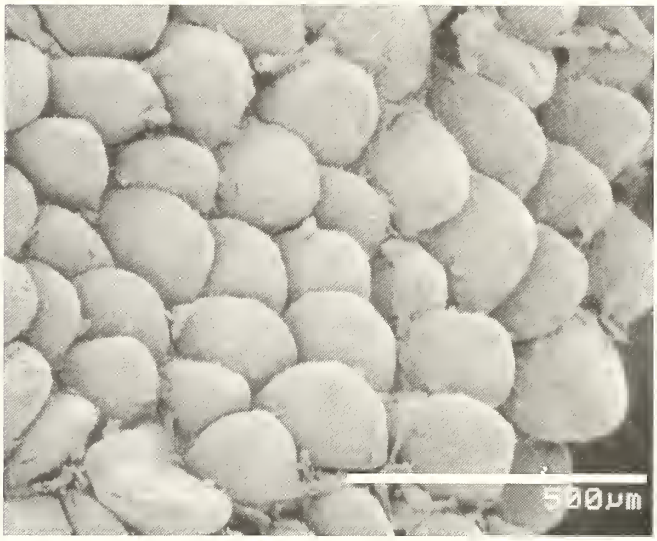

Figure 15. *Tripoplax cowani* Clark, spec. nov. Paratype (Clark 404). Dorsal girdle scales, scale bar = 500 µm.

Type locality

California, Monterey County, Carmel submarine canyon (36°45.3′N, 122°04.7′W), 954-1044 m.

Additional material

2, CASIZ 103675 and 025503, off Trinidad, Humboldt County, California (41°05′N) (*leg.* R. Talmadge, 1972; trawled, 432-720 m); 1, CASIZ 0198513 Swiftsure Bank, Washington (*leg.* I. McTaggart Cowan and D. B. Quayle, 6 September 1964; trawled, 975 m) (Paratype of *Ischnochiton*

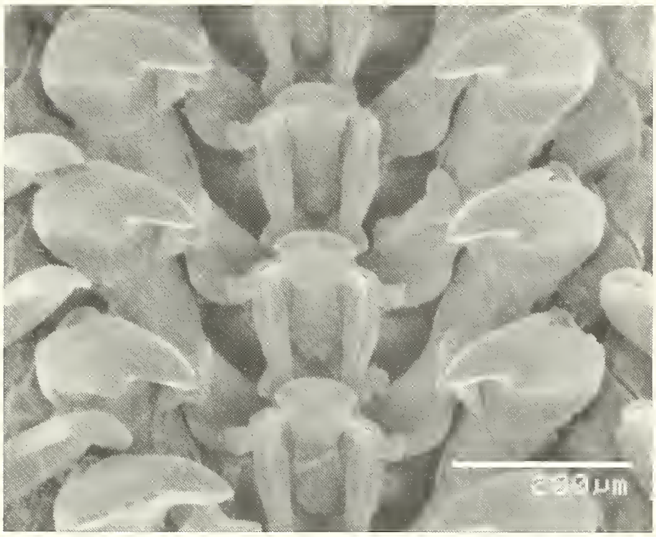

Figure 16. *Tripoplax cowani* Clark, spec. nov. Paratype (Clark 404). Radula, scale bar = 200 μm.

abyssicola Smith and Cowan, 1966); 4, RNC 2108, off Del Norte County, California (41°41.962′N, 125°00.732′W) (*leg.* R. N. Clark, 23 October 2001; trawled R/V *Miller Freeman*, 855 m; NMFS 21-0112-106).

Distribution

Tripoplax cowani has been collected from the Swiftsure Bank, Washington (48°30′N) to the type locality, Carmel Bay, California (36°45′N) at depths of 432-1044 m.

Habitat

Juveniles have been found on gravel (Smith and Cowan 1966, as *Ischnochiton abyssicola*); adults are found on large rocks and boulders.

Etymology

It is with great pleasure that I name this species after my friend and colleague, Dr. Ian McTaggart Cowan, of Victoria, British Columbia, Canada.

Remarks

The similarities between *Tripoplax cowani* and *Tripoplax abyssicola* (Figs. 17-19) in form, color, and sculpturing of the valves are remarkable, and explain why the present species has hitherto gone unrecognized. However, despite the similarities in general appearance, the two species may be readily distinguished by:

(1) Body outline, *T. cowani* is much broader than *T. abyssicola*, length 1.7 times width, compared to 2.3 in *T. abyssicola*.

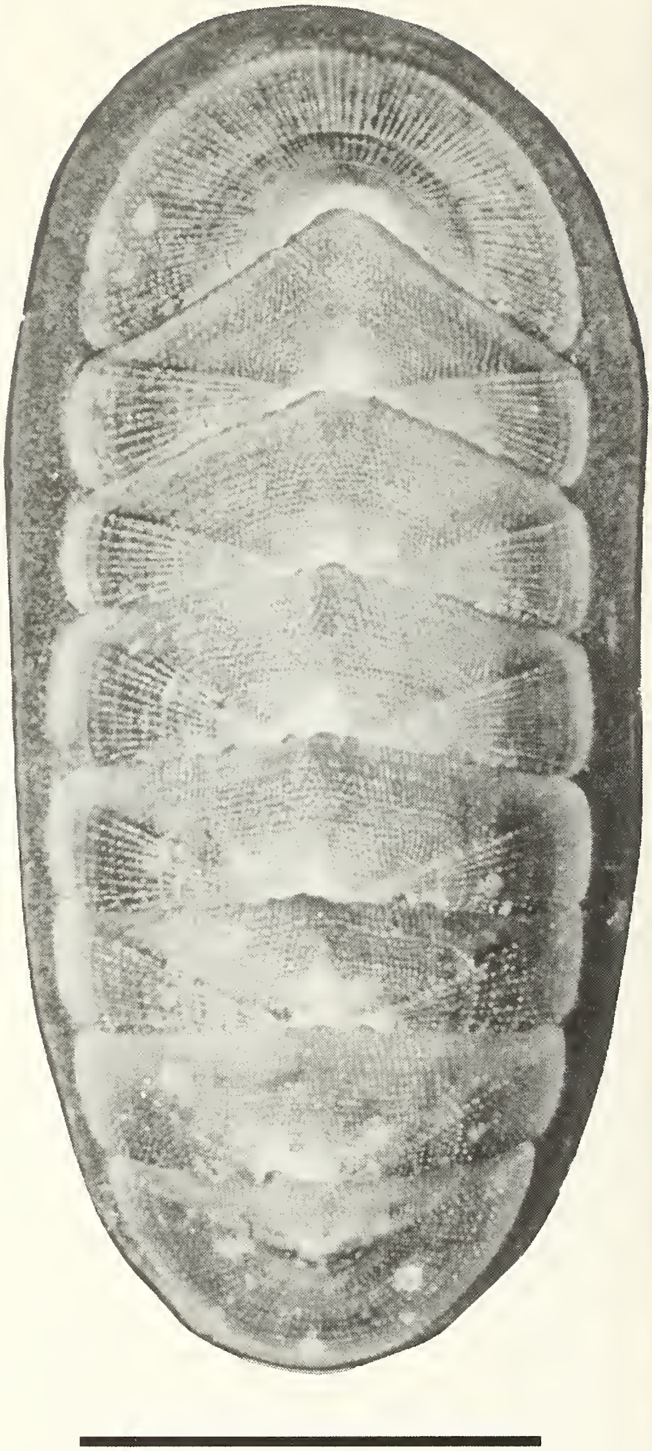

Figure 17. *Tripoplax abyssicola* Smith and Cowan, 1966. Holotype. Triangle Island, British Columbia, Canada, 870 m, scale bar = 10 mm.

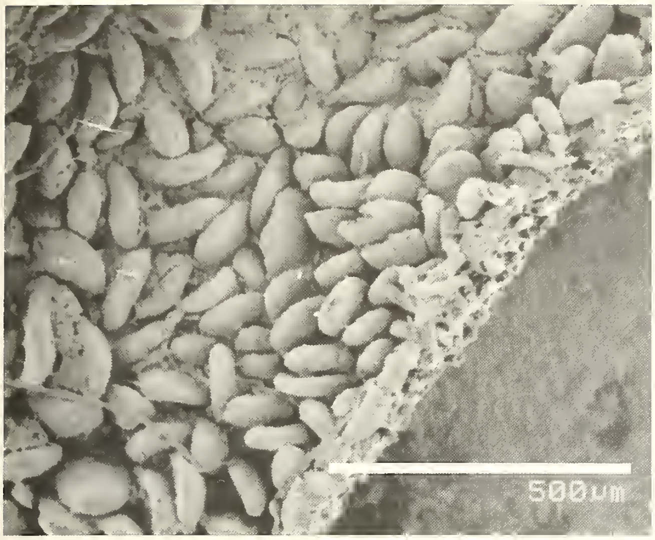

Figure 18. *Tripoplax abyssicola* Smith and Cowan, 1966. Paratype (Cowan coll., No. 5538). Triangle Island, British Columbia, 870 m, dorsal girdle scales, scale bar = 500 µm.

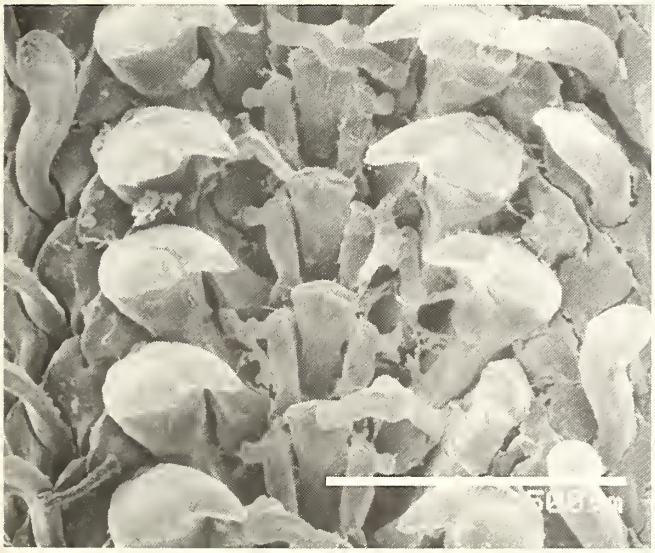

Figure 19. *Tripoplax abyssicola* Smith and Cowan, 1966; (RNC 2106, ex RNC 3106). Farallon Islands, California, 2750 m, radula, scale bar = 500 µm.

(2) Tegmental sculpturing, in animals of approx. the same size, *T. cowani* exhibits fewer and coarser radial ribs (or rows of pustules/granules). A comparison of the largest paratype of *T. cowani* (ca. 45 mm) with the holotype of *T. abyssicola* (46 mm) illustrates this well. The head valve of the *T. cowani* paratype has 70 ribs, compared to 85 in the holotype of *T. abyssicola*. The lateral areas of *T. cowani* have five to nine ribs, compared to 11-13 in *T. abyssicola*. The tail valve of *T. cowani* has 40 ribs, the tail valve of *T. abyssicola* has 65 ribs.

(3) The relative sizes of the pustules/granules on the radial ribs are quite distinct also, in animals of about the same size (45-46 mm), the pustules of *T. cowani* are 200-300 µm at about midpoint of the riblets, and form a series of about 18-19 on the lateral areas. Those of *T. abyssicola* are 100-150 µm and form a series of 28-30 on the lateral areas.

(4) Fewer, coarser riblets on central areas, 40-42 on *T. cowani*, and ≥60 on *T. abyssicola*.

(5) The dorsal girdle scales of *T. cowani* are relatively large and subtriangular in shape, reaching about 300 × 275 µm, those of *T. abyssicola* (Fig. 18) are much smaller and narrower, to about 200 × 125 µm, and are slightly curved at the tip, like diminutive surf boards.

(6) *Tripoplax cowani* has fewer ctenidia than *T. abyssicola*, 31 in a specimen 28 mm in length, compared to 37 in a 22 mm *T. abyssicola*, 36 compared to 41 in specimens 41 mm in length, and 37 for *T. cowani* and 43 for *T. abyssicola*, respectively in specimens 45 mm in length.

The geographic and bathymetric ranges of *Tripoplax cowani* and *Tripoplax abyssicola* overlap somewhat from northern California to Washington; however, *T. cowani* is generally found much shallower than *T. abyssicola*, 430-1050 m compared to 950-2750 m. *Tripoplax abyssicola* also has a much broader geographic range than *T. cowani*, extending from the western Aleutian Islands, south of Amchitka Island (51°34.14′N, 178°18.49′E) (RNC 2166; *leg.* R. Clark, 16 July 2004, trawled R/V *Sea Storm*, 478 m) to near the Farallon Islands, west of San Francisco Bay, California (38°N; RNC 2106). Range here is extended approx. 900 km west from southwest of Unalaska Island (52°36′N, 169°25′W); CASIZ 129748 (Clark 2000). The range of *T. cowani* extends from the Swiftsure Bank, off Washington (48°30′N) to Carmel Bay, California (36°45′N).

Tripoplax cowani might also be confused with *Stenosemus stearnsii* (Dall, 1902) (Fig. 20), which is smaller (to 25 mm in length) and similar in general appearance, but has broad, low, somewhat flattened, cobble-stone like sculpture on the radial areas and large, subcylindrical, curved corpuscles to 430 × 160 µm. *Stenosemus stearnsii* is found from off Clatsop County, Oregon (45°50′N) to near San Clemente Island, California (33°N) (Clark 1991), at depths of 400-700 m.

The additions of *Tripoplax cowani* and *Tripoplax calypso*, along with *Tripoplax trifida* (Carpenter, 1864), *Tripo-*

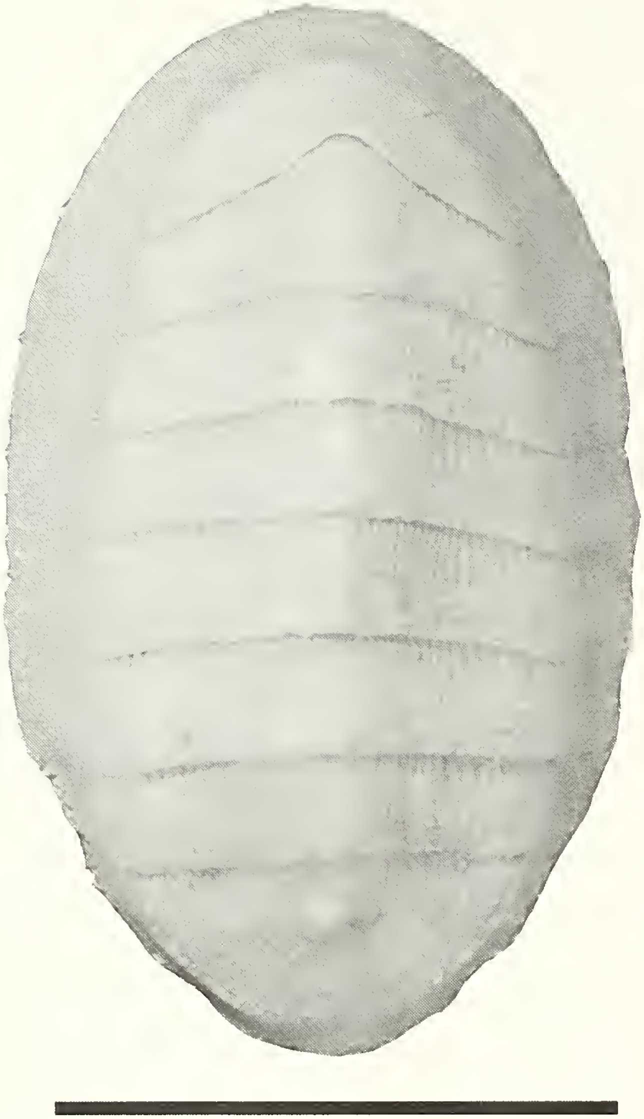

Figure 20. *Stenosemus stearnsii* (Dall, 1902). (RNC, 1583). Monterey Sea Canyon, 650 m, scale bar = 10 mm.

plax abyssicola (Smith and Cowan, 1966), *Tripoplax ima* (Sirenko, 1975), *Tripoplax allyni* (Ferreira, 1977), *Tripoplax attuensis* (Clark, 2000), *Tripoplax beringiana* (Clark, 2000) and *Tripoplax baxteri* (Clark, 2000) brings the number of known species of *Tripoplax* along the Pacific coast of North America to nine. The similar appearing *Ischnochiton regularis* (Carpenter, 1855) from northern California appears to be genetically distinct at the genus level (D. Eernisse, pers. comm., September 2007).

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The effect of sampling bias on the fossil record of chitons (Mollusca, Polyplacophora)*

Stephaney S. Puchalski[1], Douglas J. Eernisse[2], Claudia C. Johnson[1]

[1] Department of Geological Sciences, Indiana University, 1001 E. 10th St., Bloomington, Indiana 47405, U.S.A., spuchals@indiana.edu

[2] Department of Biological Science, California State University Fullerton, Fullerton, California 92834-6850, U.S.A.

Abstract: The chiton fossil record is richer than previously reported in the literature. A newly compiled database comprised of Cambrian to Pleistocene fossil chitons totals 2594 occurrences of 900 species. Of the 900, 430 are named species known only as fossils, 123 are extant species that also have a fossil record, and 247 are indeterminate taxa. Most of the database (61%) consists of fossil chiton occurrences reported from localities other than type localities. A preliminary analysis of the data using the collector curve method suggests that the chiton fossil record has not been adequately sampled by geographic regions or geologic time. The fossil record of chitons is incomplete, sporadic, and geographically limited because the sampling record has been incomplete, sporadic, and geographically limited. The current database comprises enough information to discern diversity patterns throughout geologic time, but whether the patterns are real or artifacts of sampling inadequacy remains to be investigated.

Key words: collector curve, database, sampling record, fossil record completeness, sampling adequacy

Data analysis is a fairly recent approach to investigating and discerning patterns in the fossil record (Raup 1976a, 1976b, Benton 1993, Smith 2001, Alroy *et al.* 2001, Westrop and Adrain 2001, Sepkoski 2002, Tarver *et al.* 2007, and others). Incompleteness in the data, however, including incompleteness of the fossil record itself, introduces error in interpretation of observed patterns. Both taphonomic and sampling biases cause incompleteness and affect the amount of data available for analysis (Benton 1998, Benton *et al.* 2000, Tarver *et al.* 2007), but taphonomic biases are better understood than sampling biases. Few researchers have addressed the latter issue (Tarver *et al.* 2007 and references therein). Assessment of sampling bias is essential to evaluating the adequacy of the fossil record. Understanding the causes of incompleteness allows paleontologists to use statistical methods to correct for errors in order to differentiate real patterns from apparent trends.

Despite a Cambrian to Holocene fossil record, chitons (Polyplacophora) may be less well sampled than other shell-bearing fossil fauna such as brachiopods, gastropods, bivalves, and cephalopods that have similarly long but 'good' fossil records. Although significant numbers of chiton valves (400 or more) have been recorded from some localities (Itoigawa *et al.* 1976, Bischoff 1981, Baluk 1984, Laghi 1984, Bellomo and Sabelli 1995, Cleveringa *et al.* 2000, Hoare and Pojeta 2006, Sigwart *et al.* 2007), most extinct chiton species are represented by relatively few valves that are rare compared to other taxa in an assemblage. Even when characterized as 'exceptionally abundant,' the valves are still uncommon in comparison to other taxa. For example, chiton valves were only 7% as common as bivalves found at the same Silurian localities in Gotland (Cherns 1999). Inadequate sampling thus may have affected patterns of chiton diversity and distribution reported in the literature (*e.g.*, Sepkoski 2002, Cherns 2004, Puchalski 2005). Chitons reportedly are most diverse in the Holocene (Smith 1960, Lindberg 1985, Benton 1993). About 900 modern chiton species inhabit mostly shallow coastal waters and are ubiquitous on modern rocky shores in all oceans and at all latitudes worldwide (Kaas and Van Belle 1985). In comparison, the reported chiton fossil record ranges between 256 and 368 fossil species (Smith 1960, Van Belle 1981, Eernisse 2001, Schwabe 2005), sporadically distributed through geologic time and geographically limited mostly to the North American, European, and Australia-New Zealand regions (Van Belle 1981).

This study assesses sampling bias in the chiton fossil record using the collector curve approach with a database on fossil chiton occurrences. The occurrence data also were used to show patterns in chiton diversity from the Cambrian to Pleistocene.

MATERIALS AND METHODS

Database compilation

The initial database containing 336 fossil chiton species was compiled by Eernisse (2001). The database included suborders, families, genera, type locality information, geologic period, year of publication, and museum information (*e.g.*, type kinds and numbers). The database was expanded by the first author (S.P.) to include additional occurrences of fossil chitons from the Cambrian through the Pleistocene at type and other localities. An occurrence was defined as a reported difference in geographic location of collection (latitude and longitude) or taxonomy (species). No distinction was made between geographic and taxonomic occurrences. Taxonomic differences were determined by cross-referencing the literature to account for synonymies and updating original taxonomic assignments when systematic relationships were reevaluated in later references. Taxonomic occurrences with indeterminate relationships were grouped in the genus "Indet." whether originally reported as indeterminate, unidentified, "polyplacophoran," or "chiton" regardless of whether or not the fossils were figured and/or described. The database was developed further to include modern latitude and longitude coordinates of the collecting locality, reported numbers and types of valves, authors' reasons for publication, geologic stage, and other geologic information such as lithology and general fossil associations whenever possible. The primary data sources were published reports including descriptions of fossil species, but occurrences obtained from unpublished sources (*e.g.*, online museum collection databases) also were included.

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All taxa regarded as invalid chiton fossils were excluded from this preliminary analysis of the data. For example, some Early Cambrian "polyplacophoran" fossils from China (Yü 1987) may be only superficially similar to chitons (Qian and Bengtson 1989) or may be valid chiton taxa (Yü 2001, Schwabe 2005). The fossils have an overlapping series of plates that are much smaller than other chiton valves but similarly differentiated into three types with distinct areas on the dorsal surface and shell layers consisting of 'articulamentum' and 'tegmentum.' Qian and Bengtson (1989) argue that the poorly preserved 'plates' show very few structural details and are not articulated with one another but rather represent a series of successively larger growth increments deposited on the inner side of sclerites. After restudying the specimens, Yü (2001) maintains that the fossils are indeed polyplacophorans closely related to *Gotlandochiton* Bergenhayn, 1955, *Priscochiton* Dall, 1882, *Chelodes* Davidson and King, 1884, and questionably *Glyptochiton* de Koninck, 1883. However, the first three genera are paleoloricates that are distinguished from more modern chitons in lacking articulamentum, a character that does not appear in undoubted chiton fossils until the Carboniferous (Sirenko 2006). The implication that chitons with articulamentum "gave rise" to chitons without articulamentum that then evolved into chitons with articulamentum is problematic. The remaining characters described by Yü (1987, 2001) are not necessarily exclusive to chitons. The Lower Cambrian taxa thus were rejected as valid chiton species for the purposes of this study.

The completeness of the sampling record was assessed using all fossil chiton occurrences including named species, which represent less than 17% of the entire dataset. Debates on the validity of some taxa and frequent changes in chiton systematics made it difficult to directly compare the number of named species to previous catalogues of fossil chitons (*e.g.*, Smith 1973, Van Belle 1981, Smith and Hoare 1987, Schwabe 2005). These catalogues were used in most cases to determine the validity of taxa for this study, but some catalogues are incomplete and the authors did not always agree on validity. Some species considered valid for the purposes of this study thus may not have been considered valid in previous catalogues. In cases of more recent publications, omissions from previous catalogs, or where published views conflicted regarding synonymies or validity of a particular fossil as a chiton, validity was determined by S. Puchalski using the primary literature. For example, an exhaustive literature search revealed multiple species not included in Van Belle's (1981) monograph (*e.g., Pterochiton tripartitus* Ebert, 1889 and *Pterochiton silesiacus* Ebert, 1889) that were considered to be valid chiton species. Additionally, *Chitonellus hancockianus* Kirkby, 1859, *Chitonellus antiquus* (Howse, 1848), and *Chitonellus distortus* Kirkby, 1859 named and described by Kirkby (1859) in Permian limestone at Tunstall Hill, England were listed as "no chiton" by Van Belle (1981) and rejected as polyplacophorans by Smith and Hoare (1987). The reported occurrence of these taxa was accepted as valid for this study under the name *Diadeloplax antiqua* (Howse 1848) based on Hoare and Mapes (2000), who recognized the three taxa as a single multiplacophoran species. The multiplacophorans were accepted as valid chiton taxa because Vendrasco *et al.* (2004) referred the multiplacophorans to Class Polyplacophora. The list of valid taxa and associated geographic and temporal data used in this preliminary analysis is available at: http://www.biology.fullerton.edu/deernisse/fossilchitons/. The complete database will be made available after further analysis.

The data were divided into several different groups for convenient analysis. Countries of occurrences were grouped into geographic regions (Table 1) that approximate modern continents (Tarver *et al.* 2007). Valid chiton taxa were separated into seven taxonomic groups consisting of: (1) named extinct species known only as fossils (*e.g., Lepidopleurus davolii* Laghi, 2005), (2) extinct species with names consisting of numbers or letters (e.g. *Lepidopleurus* sp. I Sulc, 1936), (3) indeterminate extinct species placed in valid genera (*e.g., Helminthochiton* sp. Plas, 1972), (4) named extinct species in indeterminate genera (*e.g.*, "Chiton" *cordiformis* Sandberger, 1845), (5) indeterminate taxa (*e.g.*, "unidentified chiton valves," Hoover 1981), (6) extant species with a fossil record

Table 1. Countries with fossil chiton occurrences grouped by geographic regions.

Geographic region	Countries
Africa	Algeria, Eritrea, Ethiopia, Morocco, South Africa, Tanzania
Asia	China, India, Japan, Malaysia, Russia, Thailand
Australasia	Australia, Borneo, Fiji, Indonesia, Marshall Islands, New Zealand, Palau
Europe	Austria, Belgium, Bulgaria, Czech Republic, Denmark, France, Germany, Greece, Hungary, Ireland, Italy, The Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Spain, Svalbard, Sweden, Ukraine, United Kingdom
North America	Bahamas, Canada, Cayman Islands, Jamaica, Mexico, Puerto Rico, United States
South America	Argentina, Brazil, Chile, Columbia, Uruguay, Venezuela

(*e.g.*, *Mopalia muscosa* (Gould, 1846)), and (7) geographic occurrences reported from localities other than type localities. The first reports of extant species were considered equivalent to type localities of extinct species. Affinities (cf., aff.), variations, and subspecies were treated as geographic occurrences and placed in group seven rather than as taxonomic occurrences in one of the other groups.

Data analysis

The collector curve approach (Weller 1952, Paul 2003, Fountaine *et al.* 2005, Tarver *et al.* 2007) was used to investigate the sampling completeness of the chiton fossil record. Assuming no decrease in effort, collector curves are exponential as discovery rates increase and become asymptotic and sigmoid when virtually every fossil taxon that has been preserved has been found (Benton 1998, Fountaine *et al.* 2005, Tarver *et al.* 2007). Collector curves, thus, are plots of the cumulative number of discoveries against some measure of collecting effort. The number of new taxa described per year and the number of fossil occurrences reported each year were used as the measure of total collecting effort. Neither approach assumes that the workers are constant. The first approach shows the rate at which workers are finding new taxa. The second approach accounts for workers finding few new taxa. In the latter case, publication history tends to move away from descriptions and into broader topics such as preservation potential and biogeography (Tarver *et al.* 2007). Publication history, thus, was assessed by categorizing the authors' primary reasons for reporting

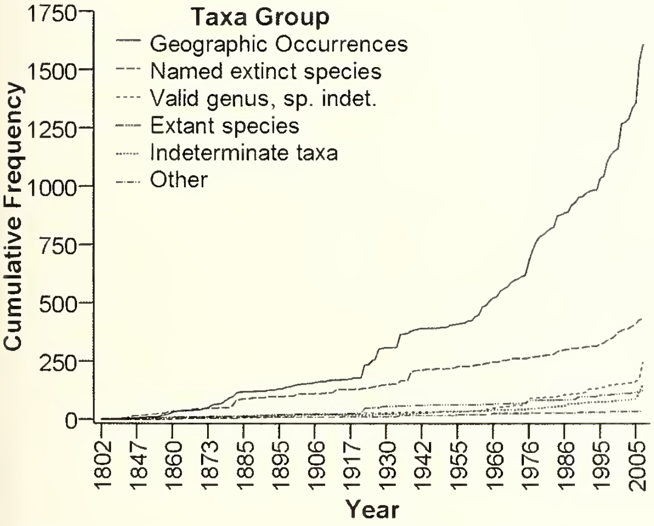

Figure 1. Collector curves for the seven groups of valid chiton taxa. The fossil record represented by the database is comprised mostly of geographic occurrences of previously described taxa discovered at localities other than type localities. The group of species with names consisting of numbers or letters and named species of indeterminate genera have been collapsed into one group labeled 'other' for clarity because these groups each represent less than 1% of the data.

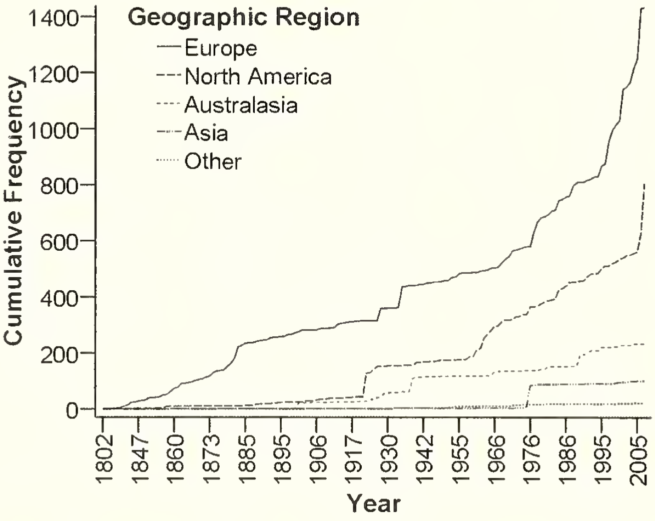

Figure 2. Collector curves for all fossil chiton occurrences reported by geographic region (see Table 1 for listing of the countries in each region). Countries in Africa and South America have been combined into one group labeled "other" for clarity because these two regions represent less than 1% of the data.

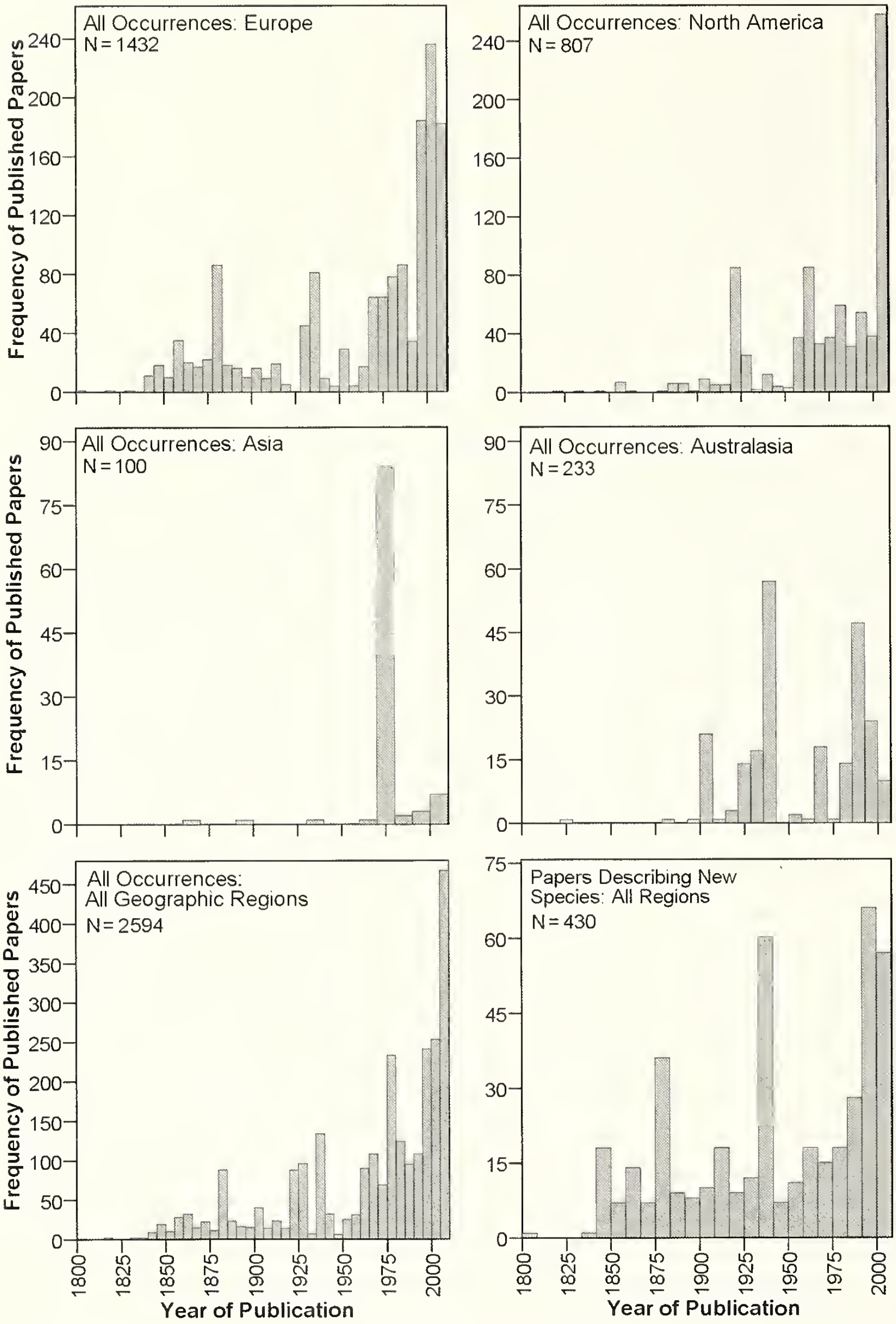

Figure 3. Histograms comparing the frequency of published papers reporting occurrences of all seven groups of fossil chiton taxa from Europe, Australasia, North America, and all geographic regions as a measure of worker effort. African and South American regions with less than 100 publications total are not shown. The publication frequency of new taxa described from all geographic regions also is shown. Note: y-axis scale varies among histograms.

the fossils based on the subject area that constituted the bulk of each publication. For example, the taxonomic group consisted of papers with systematic paleontology sections comprising most of the article. The other categories consisted of general faunal papers that did not fit into another group, biostratigraphic papers, taphonomic papers, or paleoecologic papers.

RESULTS AND DISCUSSION

At the time of analysis, the database comprised 2594 occurrences of 900 chiton taxa. Of the 900 taxa, 430 are valid fossil species named and described from 1802 through 2007, 123 are extant species with a fossil record, and 247 are indeterminate species. The 900 taxa are placed in 95 genera, 31 families, 9 suborders, and 4 orders of the Class Polyplacophora. In comparison, Van Belle (1981) listed 250 named and 49 indeterminate fossil species and a few extant species with a fossil record. Smith's (1960) compilation included 293 named and unnamed species known only as fossils and 59 extant species with a fossil record. Smith and Hoare (1987) reported 153 Paleozoic named species and 23 indeterminate taxa. Schwabe (2005) reported 368 named species known only as fossils. Despite the importance of these previous fossil catalogues, named fossil and extant species combined represent only about 21.3% of all 2594 fossil occurrences in the current database. Most of the data (61.0%) consists of previously identified species that occurred at localities other than type localities. The large numbers of geographic occurrences suggest that the fossil record is richer than previously indicated in the literature.

The fully exponential pattern of the collector curves indicates that sampling of fossil chitons has been inadequate for all taxonomic groups (Fig. 1) and geographic regions (Fig. 2). The stepped Asian curve and roughly asymptotic Australasian curves potentially suggest that the sampling records are complete for these regions. The combined African-South American collector curve also appears relatively flat, but this is partly due to limited data and partly due to the scale of the graph required to show the Australasian, European, and

North American curves that represent the majority of data. However, flattening of collector curves also may be caused by decreased collecting effort (Tarver *et al.* 2007). As a measure of collecting effort, decreased frequency in the number of published papers suggests that flattening of the Asian and Australasian collector curves are due to decreased efforts rather than failure to find new species (Fig. 3). In comparison, the increased frequency of papers on European occurrences suggests collecting efforts have risen in the last few decades. The corresponding European collector curve has not reached an asymptote (Fig. 2), indicating continued high rates of discovery of new taxa. In North America, collecting efforts have remained relatively high from 1960 to the present but the collector curve still is in the exponential phase, similarly indicating high discovery rates of new taxa. The incomplete sampling records thus result from heterogeneity in collecting effort focused on fossil chitons.

The frequency of papers describing new species from all seven geographic regions shows a fairly steady increase from 1950 to the present (Fig. 3). However, the data also suggest there is much information in the fossil record of chitons that has yet to be tapped. Most papers were published for the purposes of describing new taxa (Fig. 4). Taxonomic reports comprise most of the reported occurrences (N = 2238, 86.2%), although some occurrences were reported as part of general fauna (N = 205, 7.9%) or biostratigraphic studies (N = 95, 3.7%). Few fossil chitons were reported as part of taphonomic (N = 5, 0.2%) or ecologic (N = 51, 2.0%) studies. The implication is that the range of research on fossil chitons still is mostly in the discovery phase and has yet to broaden.

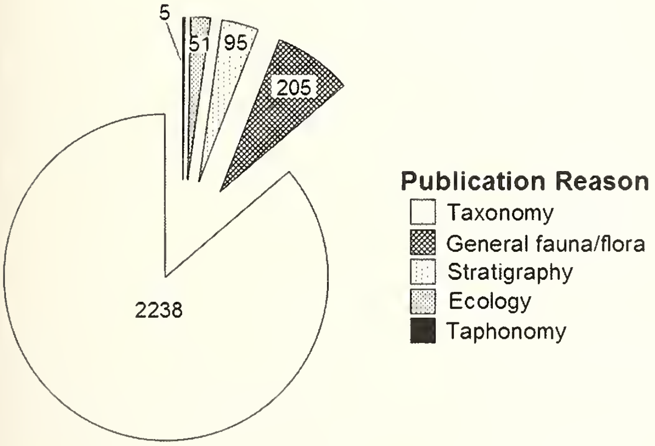

Figure 4. Pie chart showing the distribution of the reasons for publication of papers reporting occurrences of fossil chitons. Reasons were determined by the subject areas that constituted the bulk of each publication: taxonomy, systematic descriptions; biostratigraphy, temporal correlations; paleoecology, paleoecological analyses; taphonomy, taphonomic analyses; general fauna/flora, papers not fitting into previous categories. Numbers shown in each slice indicate the number of publications in each category.

Although the collector curves indicate inadequate sampling of all seven groups of fossil chiton taxa through the Phanerozoic (Fig. 5), the data may be sufficient for some geobiological studies depending on the geologic time and/or geographic region being investigated. Some geologic time periods and geographic fossil records of chitons are more complete than others due to the heterogeneity in collecting effort. For example, most fossil species occur in the Carboniferous and Cenozoic (Eocene and Miocene to Pleistocene, Fig. 6). Although the shapes of the Cenozoic and Paleozoic curves are similar among groups of taxa, active research and focused collecting efforts can be attributed to a limited number of researchers that have contributed to the increases in both cases. Richard Hoare, as author or coauthor, has described thirty-one new Carboniferous chiton species, resulting in a more complete record for the period. The Cenozoic increase may be attributed to the more complete sampling of Holocene biota referred to as the 'pull of the Recent' (Raup 1979, Foote 2000, Alroy *et al.* 2001, Peters and Foote 2001). However, Bruno Dell'Angelo, as author or coauthor, accounts for 242 or 11.8% of the Cenozoic occurrences, most in the Mediterranean, suggesting that focused collecting effort has resulted in a more complete Cenozoic record for the European region. In comparison, the Mesozoic collector curves suggest severely inadequate sampling of chitons for that period.

Temporal gaps shown by this analysis do not necessarily equate to non-existence of chitons in past ecosystems. Although the non-logistic nature of the collector curves indicates that at least some gaps in the current dataset are due to sampling bias, the generally poor preservation states of most fossils indicate that taphonomic biases also may have been a contributing factor. Paleoecologic data show that Paleozoic chitons mostly inhabited shallow coastal environments similar to the settings inhabited by most modern chiton species (Dunlop 1915, 1922, Frederickson 1962, Smith and Toomey 1964, Kues 1978, Yancey and Stevens 1981, Gerk and Levorson 1982, Hoare and Smith 1984, Debrock *et al.* 1984, Farrell 1992, Vendrasco 1999, Cherns 1999, Hanger *et al.* 2000, Hoare 2001, Cherns 2004, Vendrasco and Runnegar 2004). There is no reason to assume that the preservation potential of chitons in the past differed greatly from that in modern environments. Most modern chitons tend to live in intertidal or shallow subtidal erosional environments that are rarely preserved even where fossil deposits are extensive (*e.g.*, California Miocene). Modern chitons are more rarely found living in relatively deep water or on muddy bottoms, but species diversity in such cases is much lower relative to shallow water communities. Fossil chitons interpreted to have lived on muddy bottoms or in deeper water are rare, but have been reported (Hoare *et al.* 1972, Lang and Chlupac

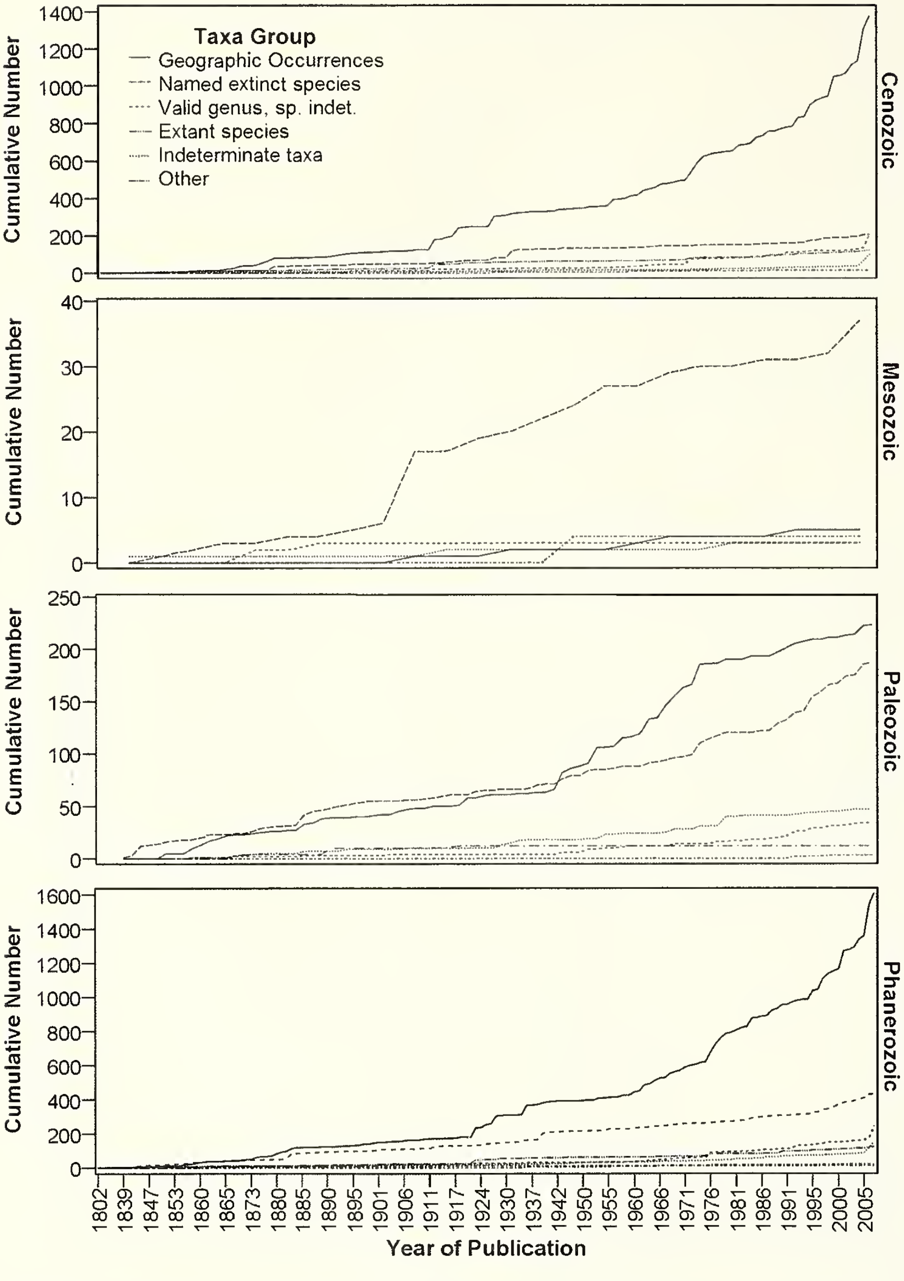

Figure 5. Collector curves for each taxa group of fossil chiton occurrences reported by geologic era and for Phanerozoic. Not all seven groups have been reported from all three eras.

1975, Richardson 1980, Dell'Angelo and Palazzi 1994, Palazzi and Villari 1994, Goedert and Campbell 1995, Squires and Goedert 1995, Remia and Taviani 2005, Kiel and Goedert 2006). As with modern settings, diversity appears to be lower relative to the shallow water assemblages.

Observed changes in chiton diversity through time (Fig. 6) do not correlate to degrees of preservation or skeletal completeness. In general, changes in species numbers through geologic time indicate chitons were affected by mass extinctions (Fig. 6). For example, decreased numbers of species in the Paleocene imply that chitons were affected by the end-Cretaceous mass extinction. The species numbers increased in the Eocene following an apparent slow recovery through the Paleocene. The mean number of species per occurrence (≈ locality) used as a proxy for diversity suggests that chiton diversity has remained relatively constant through the Phanerozoic (Fig. 6). Pleistocene diversity is not significantly greater than Eocene or Late Permian diversity, for example. Whether these patterns are artifacts of the sampling inadequacy or real trends remain to be investigated. Continued active collection and study of fossil chitons should be encouraged because the non-logistic nature of the collector curves suggest that many more fossil chiton species remain to be found and described. Recent discoveries have been instrumental in demonstrating that Paleozoic chitons were more diverse in form than modern chitons (*e.g.*, Pojeta *et al.* 2003, Vendrasco *et al.* 2004). Further analysis will investigate large-scale evolutionary and ecological patterns in the data with the goal of assessing the fidelity of the chiton fossil record after correcting for the sampling bias indicated in this preliminary analysis.

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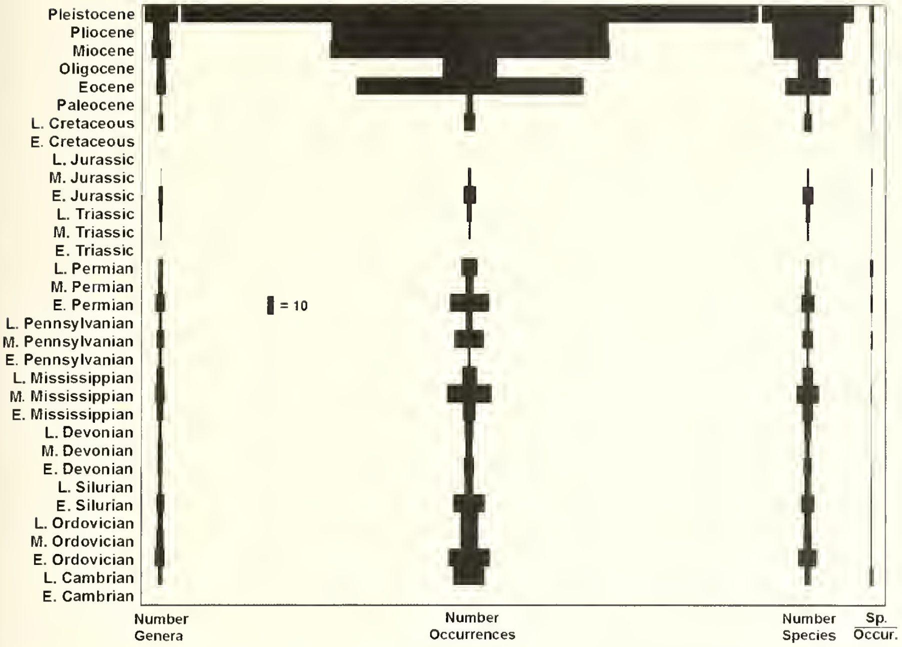

Figure 6. Diagram showing numbers of genera, occurrences, and species by epoch. Sp./occur., number of species per occurrence. Bar width of $N = 10$ shown for scale.

Dell'Angelo contributed an extensive list of references on chitons. R. Hendrickson provided critical proof reading. Comments by J. Pojeta and an anonymous reviewer greatly improved the original manuscript.

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Fertilization biology and the evolution of chitons*

John Buckland-Nicks

St. Francis Xavier University, Antigonish, Nova Scotia B2G 2W5, Canada, jbucklan@stfx.ca

Abstract: Studies of gamete structure and fertilization biology have revealed much about the phylogeny of molluscs. Recent studies of fertilization in chitons support the view that the basal order of chitons, the Lepidopleurida, fertilize eggs, as most molluscs do, by fusing the entire sperm with the egg and transferring chromatin, mitochondria, and centrioles into the egg cytoplasm. However, current evidence suggests that all members of the order Chitonida inject only chromatin into the egg. These chitons, which include the controversial family Callochitonidae, share a series of synapomorphic characters based on their fertilization biology that makes them unique. Current evidence suggests that Callochitonidae are basal to this order and sister taxa to the remaining Chitonida, which have been divided into two suborders, the Chitonina and Acanthochitonina. New evidence indicates that Chitonina have at least two different mechanisms for penetrating the egg. One group of species has pores in the egg hull (*e.g.*, *Chaetopleura apiculata* (Say in Conrad, 1834) and *Stenosemus albus* (Linnaeus, 1767), whereas a second group has a continuous dense layer on the surface of the egg hull that is digested by the sperm (*e.g.*, *Rhyssoplax tulipa* (Quoy and Gaimard, 1835) and *Stenoplax conspicua* (Pilsbry, 1892)). However, the genus *Ischnochiton* Gray, 1847 appears to be polyphyletic, as several species have distinctive characters that typify other genera or families. In particular, this genus needs to be re-evaluated using modern morphological and molecular methods. All of the Chitonina have spiny-hulled eggs with narrow bases and are quite different from the second suborder, Acanthochitonina, which is characterized by large-hull cupules with wide bases. Within Acanthochitonina, some species have open-hull cupules, whereas most have closed ones. Open-cupule species lack micropores in the hull for sperm entry, whereas several closed-cupule species exhibit micropores between hull cupules. These features of the egg are accompanied by alterations in sperm structure, such as position of the mitochondria and structure of the basal body, acrosome, and flagellum. Knowledge of the gamete structure of individual species and their fertilization biology, as demonstrated here, provides a different series of characters that can help avoid mistakes that are inherent during early development of new methods, such as molecular analyses. New details of fertilization biology have made it possible to revise preliminary analyses and provide an updated phylogeny of chitons, which differs in some important respects from other recent publications.

Key words: Polyplacophora, egg, sperm, cladistics, phylogeny

We are coming to rely more and more on molecular analyses to accurately explain phylogenetic relationships (Peterson and Eernisse 2001, Okusu *et al.* 2003, Eernisse and Peterson 2004, Smith *et al.* 2004). Nevertheless, errors and inconsistencies do occur, making it useful to have a series of checks and balances in place based on a different set of characters. Prior to 1984, chiton taxonomy relied heavily on the structure of shell valves, spicules, scales, and girdle processes in order to distinguish between taxa (Smith 1960, Van Belle 1983, Kaas and Van Belle 2003), but it became apparent that some of these characters had evolved more than once by convergent evolution, which introduced some problems in classification.

Eernisse (1984) first suggested the use of egg structure and gill placement as additional characters, and Sirenko (1993) included these and produced a revised classification that divided chitons into two orders, Lepidopleurida and Chitonida, with the latter comprising two suborders, Chitonina and Acanthochitonina. A cladistic analysis by Buckland-Nicks (1995), which added characters for gamete biology at fertilization, supported this classification, differing only in positions of certain families within each suborder. In that analysis the family Callochitonidae came out as basal within Chitonina or as sister taxon to this suborder within Chitonida. *Cyanoplax* Gould, 1849 (= *Lepidochitona* Gray, 1821) did not place within Tonicellidae, as suggested by Sirenko (1993), but rather came out as a sister taxon to Acanthochitonidae. More recently Okusu *et al.* (2003) undertook a combined molecular and morphological study that provided the first detailed analysis of this kind. In general, their paper agreed with Sirenko (1993) and Buckland-Nicks (1995), but it differed in some important aspects. For example, Okusu *et al.* (2003) placed *Callochiton* Gray, 1847 (Callochitonidae) within Lepidopleurida, not in Chitonida. Furthermore, there were inconsistent results from their different molecular analyses (16sRNA, COI, and histone H3) for both *Schizochiton* Gray, 1847 and *Lepidozona* Pilsbry, 1892 (Okusu *et al.* 2003: figs. 2, 3, 4, pp. 288-291). Most previous classifications, including the combined morphological dataset of Okusu *et al.* (2003: fig. 8, p. 295), placed *Lepidozona* close to *Stenoplax* in Ischnochitonidae. The genus *Ischnochiton* Gray, 1847 consistently came out as being a polyphyletic taxon, with some species of the genus turning up in different families (Okusu *et al.* 2003). Furthermore, they did not find support for a monophyletic Acanthochitonina, suggesting instead that it comprised two or more paraphyletic clades.

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A preliminary re-analysis of morphological data (Buckland-Nicks 2006) once again placed Callochitonidae outside the Lepidopleurida, either as sister taxon to Chitonida or basal within it, as suggested previously (Buckland-Nicks and Hodgson 2000). More recently scientists have begun looking at other potential characters for distinguishing taxa, such as mineral composition of the radula (Brooker *et al.* 2006) and the gene sequence coding for hemocyanin protein (Lieb *et al.* 2006).

This paper seeks to further elucidate the morphology of sperm and eggs at fertilization in selected key taxa and thereby provide a focus for discussion of some of these new ideas. Furthermore, previous morphological data are reanalyzed and updated with current information, providing some new insights into chiton phylogeny, which are remarkably consistent with those achieved by completely new types of analysis (Lieb *et al.* 2006). Ideally, all of these analyses should be combined to provide the most comprehensive test of phylogenetic relationships.

MATERIALS AND METHODS

Specimens

Specimens were collected from various sites around the world (see Table 1). Species of the following families are represented (terminology following Kaas *et al.* 2006 and Eernisse *et al.* 2007): Leptochitonidae (= Lepidopleuridae), Callochitonidae, Ischnochitonidae, Chitonidae, Acanthopleuridae, Lepidochitonidae, Acanthochitonidae, and Mopaliidae. Several minor families that have not been included because insufficient data are available on their reproductive biology include (terminology following Sirenko 2006): Ferreiraellidae, Nierstraszellidae, Callistoplacidae, Loricidae, Hemiarthridae, Choriplacidae, Schizochitonidae, and Cryptoplacidae.

Light and electron microscopy

Fixation for transmission electron microscopy (TEM) and scanning electron microscopy (SEM) followed methods of Buckland-Nicks and Hodgson (2000). Individual chitons were isolated in petri dishes and induced to spawn by adding sperm to each dish. Egg-laying females were removed from

Table 1. A list of chiton species collected for the study and arranged in alphabetical order (all coordinates are approximate). Where a new genus has been assigned, the old genus is shown in brackets; subgenera are not shown.

Species	Collection site/year	Latitude, longitude	Collected by
Acanthopleura granulata (Gmelin, 1791)	Trinidad 2001	10°40′N, 61°39′W	J. Buckland-Nicks (J.B.-N.)
Acanthochitona viridis (Pease, 1872)	Oahu, Hawaii 1987	21°18′N, 158°09′W	J.B.-N.
Callochiton dentatus (Spengler, 1797)	East London, S. Africa 1999	33°03′S, 28°03′E	J.B.-N. and A. Hodgson
Chaetopleura apiculata (Say in Conrad, 1834)	Florida 2004		Gulf Specimen Co., Florida
Cryptochiton stelleri (von Middendorff, 1847)	San Juan Is., Washington 1988	48°28′N, 122°54′W	J.B.-N.
Cyanoplax (Lepidochitona) dentiens (Gould, 1846)	San Juan Is., Washington 1990	48°28′N, 122°54′W	J.B.-N. and D. Eernisse
Cyanoplax (Lepidochitona) fernaldi Eernisse, 1986	San Juan Is., Washington 1990	48°28′N, 122°54′W	J.B.-N. and D. Eernisse
Deshayesiella curvata Carpenter in Pilsbry, 1892	Vostok Bay, Russia 1997	42°53′N, 132°44′E	B. Sirenko
Hanleya hanleyi W. Bean in Thorpe, 1844	Lurcher Bank, Canada 1994	43°15′N, 65°30′W	R. Mayhew
Leptochiton asellus (Gmelin, 1791)	Bergen, Norway 2006	60°20′N, 5°11′E	J.B.-N and C. Schander
Leptochiton assimilis Thiele, 1909	Vostok Bay, Russia 1997	42°53′N, 132°44′E	B. Sirenko
Leptochiton rugatus (Pilsbry, 1892)	Vostok Bay, Russia 1997	42°53′N, 132°44′E	B. Sirenko
Mopalia muscosa (Gould, 1846)	San Juan Is., Washington 1990	48°28′N, 122°54′W	J.B.-N. and D. Eernisse
Nuttallina californica (Nuttall MS, Reeve, 1847)	S. California 1993		Sea Life Supply, California
Radsia (Chiton) nigrovirescens de Blainville, 1825	Eastern Cape, S. Africa 1999	34°16′S, 18°40′E	J.B.-N.
Rhyssoplax (Chiton) tulipa Quoy and Gaimard, 1835	East London, S. Africa 1999	33°03′S, 28°03′E	J.B.-N. and A. Hodgson
Stenoplax conspicua (Pilsbry, 1892)	S. California 1993		Sea Life Supply, California
Stenosemus (Ischnochiton) albus (Linnaeus, 1767)	Bergen, Norway 2006	60°20′N, 5°11′E	J.B.-N. and C. Schander

dishes, cleaned, and replaced in clean dishes with filtered sea water. Eggs, and sometimes fertilization events, were observed with Nomarski optics. Overnight primary fixation in ice cold 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) in filtered sea water with 0.1M sucrose was followed by secondary fixation with 2% osmium tetroxide for 1 h in the same buffer. Fixed eggs were rinsed in distilled water and dehydrated in an ethanol series to 100%. Batches of eggs were divided into equal aliquots and processed separately for TEM and SEM.

For TEM, ethanol was replaced with propylene oxide and then eggs were infiltrated with either TAAB 812/Araldite or Spurrs/Epon. Samples in pure resin were left all day before baking in a 60 °C oven for two days. Thick and thin sections were cut with a diamond knife (Diatome, Switzerland). Thin sections were picked up on naked 150 mesh copper grids and stained sequentially with aqueous uranyl acetate (20 min) and lead citrate (5 min) with extensive washing with degassed distilled water between stains and after staining. Stained sections were examined in a Philips TEM 410 operated at 80 kV.

Eggs destined for SEM were aspirated into Teflon flo-thru vials (Pelco) before critical point drying, mounting on stubs using double-sided carbon tabs, and coating with gold in an SPI-Module Sputter Coater. Stubs were examined in a JEOL JSM 5300 SEM and photographed.

RESULTS

Gamete structure and fertilization in Leptochitonidae (Order Lepidopleurida)

The sperm of *Leptochiton rugatus* (Pilsbry, 1892) and *Leptochiton assimilis* Thiele, 1909 are essentially similar in structure to *Leptochiton asellus* (Gmelin, 1791) (Hodgson *et al.* 1988), having a bullet-shaped nucleus capped by an elongate acrosome cone about 3 μm in length with subacrosomal material (Figs. 1A, 1C, 2A). The proximal and distal centrioles are central and can be distinguished as separate entities although connected by flocculent material (Fig. 1B). They are surrounded by five or six large, spherical mitochondria and give rise to a central flagellum (Figs. 1A, 2A). Sperm of the lepidopleurids *Deshayesiella curvata* Carpenter in Pilsbry, 1892 and *Hanleya hanleyi* W. Bean in Thorpe, 1844 are essentially similar in terms of centrioles and mitochondria. However, in these sperm, as an extension of the nucleus, there is a short nuclear filament less than 2 μm in length, which is capped by a smaller acrosome about 1 μm long (Figs. 1D, 2B).

The eggs of *Leptochiton asellus* have a smooth jelly coat without pores (Figs. 1E, 1F, 3A), whereas those of *Deshayesiella curvata* have been shown to have large pores in the jelly coat (Pashchenko and Drozdov 1998), similar to those of *Callochiton dentatus* (Spengler, 1797) (= *Callochiton castaneus* Wood, 1815) (Fig. 3B). Observations of fertilization in *L. asellus* within ten minutes of exposure of eggs to sperm revealed that the sperm had digested the jelly coat and breached a large hole in the vitelline layer, coming to rest in the perivitelline space (Figs. 1E, 1F). This is the first time in chitons, and specifically in lepidopleurids, that the entire sperm has been shown to penetrate below the vitelline layer.

Gamete structure and fertilization in Callochitonidae (Order Chitonida)

The sperm of *Callochiton dentatus* is unlike lepidopleurid sperm because it has a nuclear filament greater than 3 μm in length, tipped by a highly reduced acrosome (Fig. 2C). Furthermore, the centrioles are fused into a slightly acentric basal body and the five mitochondria are not all spherical (Fig. 2C, 4A). This description would apply to virtually all sperm of Chitonida but none of Lepidopleurida, so far described.

The eggs of *Callochiton dentatus* are similar to those of *Deshayesiella curvata* with open pores in a thick jelly coat (Fig. 3B). Each pore coincides with a depression in the egg membrane. At fertilization the sperm nuclear filament bridges the gap between the vitelline layer and egg membrane, thus replacing the acrosomal process that characterizes Lepidopleurida and other molluscs. This is significant because in *C. dentatus*, when the acrosome digests the vitelline layer, only a tiny pore is made (Fig. 4B), which does not permit the fertilization cone to raise up and engulf the entire sperm, and it remains below the vitelline layer (Figs. 4B, 4D). Current evidence suggests that sperm mitochondria and centrioles are left on the surface of the egg.

Gamete structure and fertilization in Chitonina (Order Chitonida)

The sperm of all Chitonina have very acentric basal bodies in which the proximal centriole is positioned perpendicular and lateral (on the mitochondrial side) to the distal centriole in a fused mass (Figs. 2D, 4E, 5A, 5B). The basal body is usually in line with one side of the nucleus, with the mitochondria reduced in number to 3 or 4 on the other side. As usual, the annulus binds the distal centriole to the plasma membrane but extending from it, along the plasma membrane, mainly on the side containing the mitochondria, is a dense thickening (Fig. 2D, 4E, 5A, 5B). In addition to basal mitochondria, lateral mitochondria are present in sperm of some Chitonina, such as *Ischnochiton*, *Stenoplax*, and *Lepidozona* (Fig. 5B). Glycogen rosettes are visible in the space between mitochondria and centrioles (Figs. 2D, 4E, 5A, 5B).

All Chitonina have eggs with elaborate hulls raised into

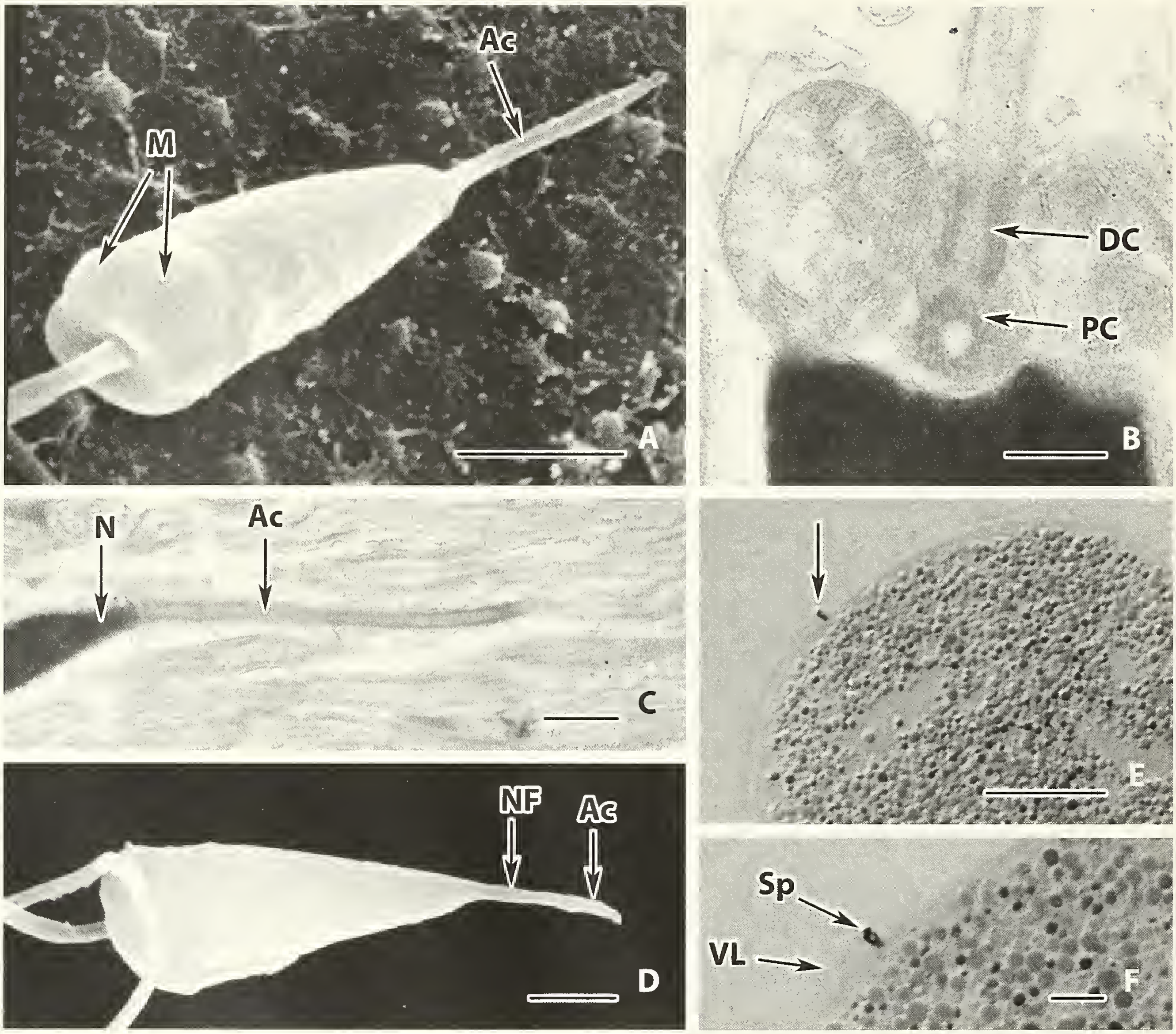


Figure 1. A, Sperm of *Leptochiton asellus* viewed with SEM. Note anterior elongate acrosome (Ac) and mitochondria (M) posterior to nucleus. Scale bar = 1 μm. B, TEM of base of sperm of *Leptochiton rugatus* showing separate proximal centriole (PC) and distal centriole (DC), adjacent to spherical mitochondrion and dense nucleus. Scale bar = 0.3 μm. C, Sperm acrosome cone (Ac) and tip of nucleus (N) of *Leptochiton assimilis*, viewed with TEM. Scale bar = 0.5 μm. D, Sperm of *Deshayesiella curvata* viewed with SEM. Note nuclear filament (NF) and acrosome (Ac). Scale bar = 1.5 μm. E, Light micrograph of 1 μm section of egg of *Leptochiton asellus* showing that entire sperm has breached vitelline layer (arrow). Scale bar = 35 μm. F, Part of E magnified to show penetrating sperm (Sp) beneath vitelline layer (VL), prior to entry into the egg. Scale bar = 7 μm.

a series of spines with narrow bases ranging in size from 5 to 30 μm (Figs. 3C, 3D, 6A-D). Two main types of fertilization have been observed. The first type involves penetration of open pores in the hull (Fig. 7B); and the second type involves sperm digestion of a thin dense layer covering the hull (Figs. 7D, 7E).

Mechanism 1: Fertilization via open pores in hull

Stenosemus albus (= *Ischnochiton albus*) and *Chaetopleura apiculata* have open pores in the egg hull, ranging in size from 1 to 4 μm, at the base of the spines. Within about thirty seconds of sperm release into a beaker of eggs, some sperm locate and penetrate these pores with their

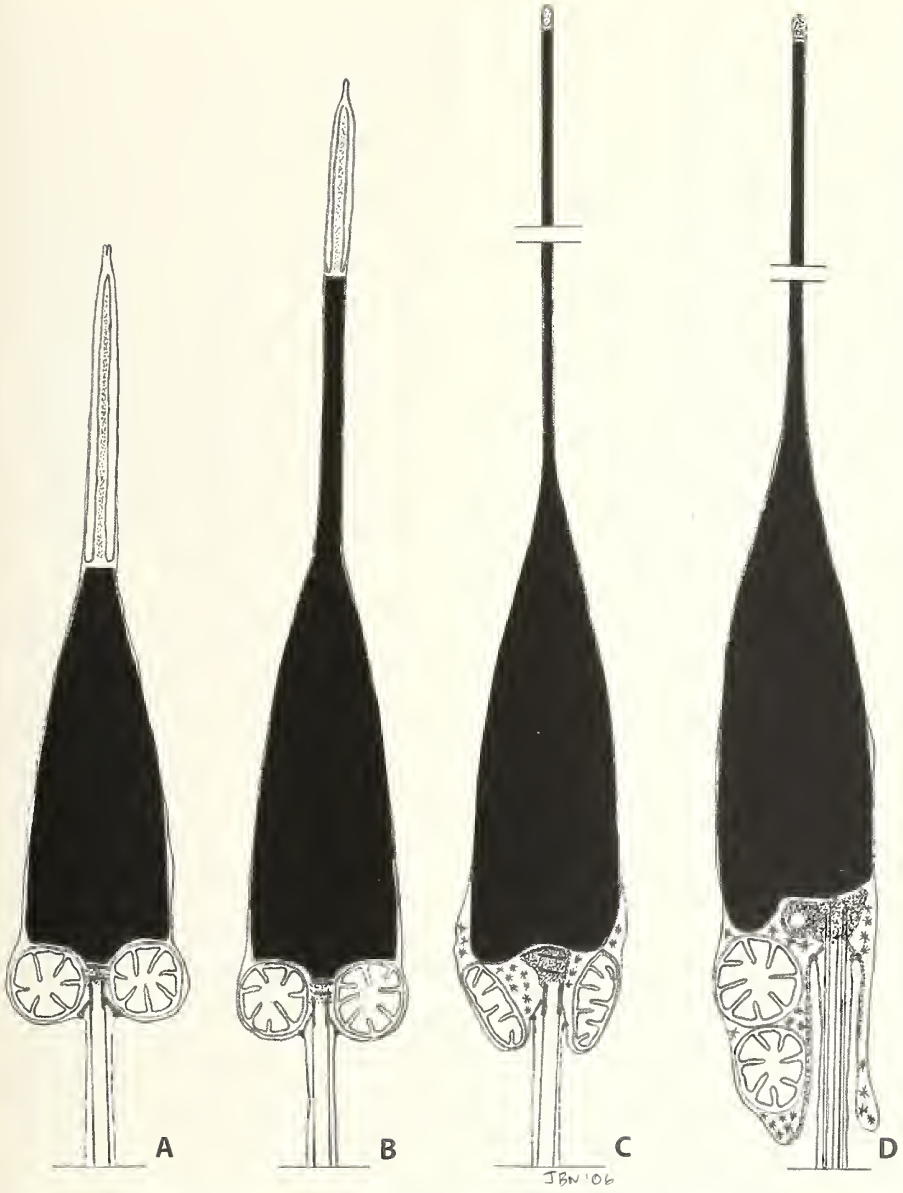


Figure 2. A, Sperm of *Leptochiton asellus*; B, sperm of *Deshayesiella curvata*; C, sperm of *Callochiton dentatus*; and D, sperm of *Chiton tuberculatus*.

anterior filament (Fig. 7B). The pores provide sperm direct access to the vitelline layer, which is digested by the acrosome before fusion occurs between sperm and egg membranes.

Hull spines of *Chaetopleura apiculata* eggs are complex with many branches off each spine (Fig. 6D). Overlapping branches between spines create complex channels that the sperm must negotiate in order to reach the base of the spines, where the pores are located. In *C. apiculata* the pores can be large enough to admit entire sperm, which sometimes gain direct access to the vitelline layer, before penetrating the egg (Buckland-Nicks and Brothers 2008).

In *Stenosemus albus* the hull comprises long spines with recurved tips (Fig. 3C). Pores are arrayed alongside the junctions of hexagonal bases, around the perimeter of each base (Fig. 7B).

Mechanism 2: Fertilization via a dense layer on hull

Rhyssoplax tulipa (= *Chiton tulipa* Quoy and Gaimard, 1835) and *Stenoplax conspicua* both have a continuous dense layer overlying the hull. This dense layer is invariably digested by the acrosome reaction on contact with the hull (Figs. 7D-E) and has been observed in numerous thick sections as well as some thin sections of both species. *Radsia nigrovirescens* (= *Chiton nigrovirescens* de Blainville, 1825) is unusual among other Chitoninae examined in having pores in the hull (Fig. 7A) although it is not known if these are used by the sperm at fertilization. Typically, brooding chitons have reduced spines or cupules, but specimens of *R. nigrovirescens* have long, simple spines with hooked tips (Fig. 3D), more like those of *Stenosemus albus*, than those of other Chitoninae.

Acanthopleura granulata (Gmelin, 1791) has unusual polymorphic spines on the egg (Fig. 6C). Some spines are short and bifurcating at the tip whereas others are intermediate or longer in length and have a scaly appearance (Fig. 6C). This egg does not appear to have pores in the hull and the sperm penetrate directly, in the same way as they do in *Stenoplax conspicua* and *Rhyssoplax tulipa*.

Gamete structure and fertilization in Acanthochitonina (Order Chitonida)

Gamete structure and fertilization in Acanthochitonina is broadly similar to that in Chitonina. However, all sperm of Acanthochitonina can be distinguished by having anterior mitochondria as well as basal and lateral ones, numbering 7 or 8 in total. Below the annulus, a fibrous complex is found

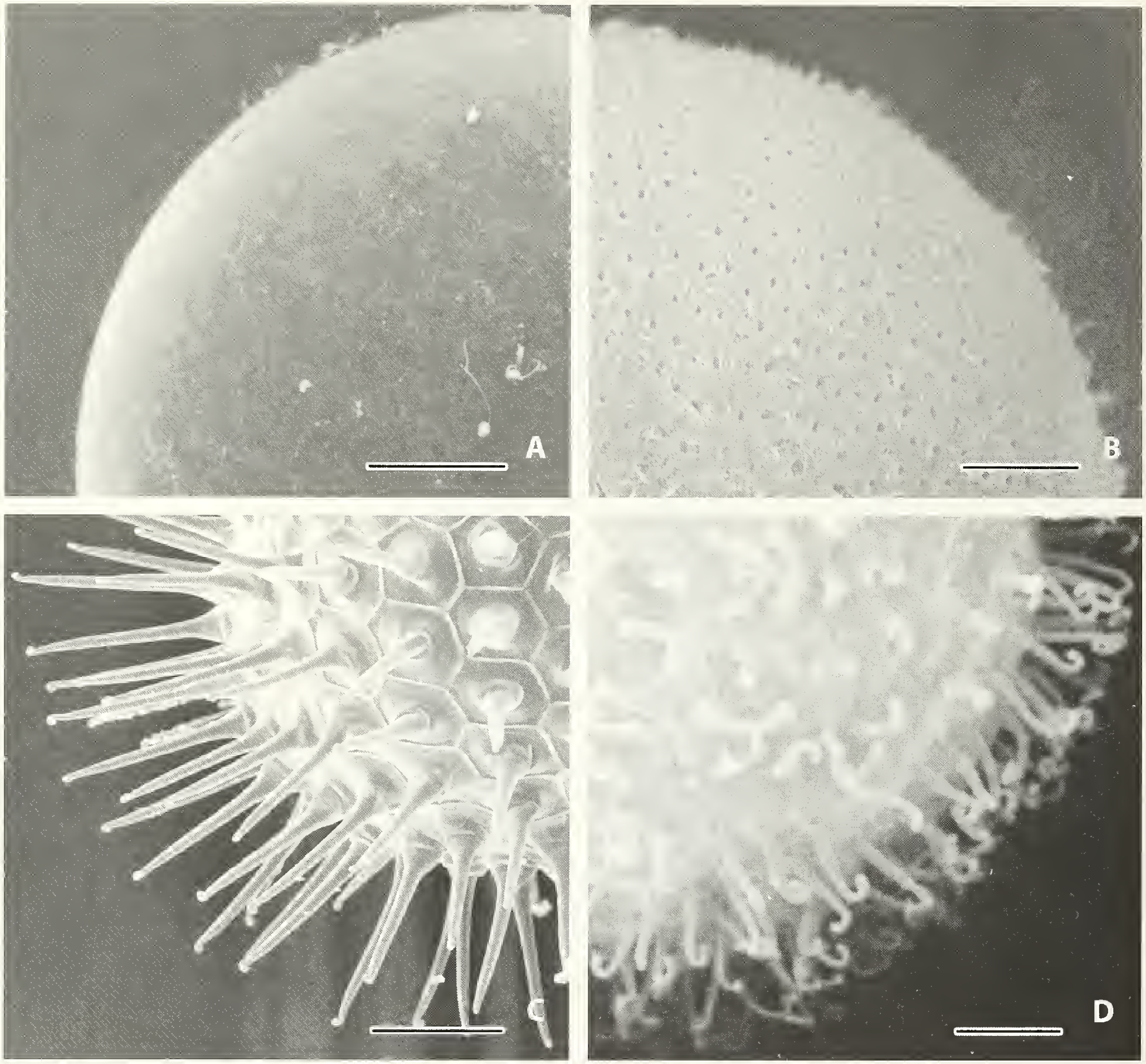
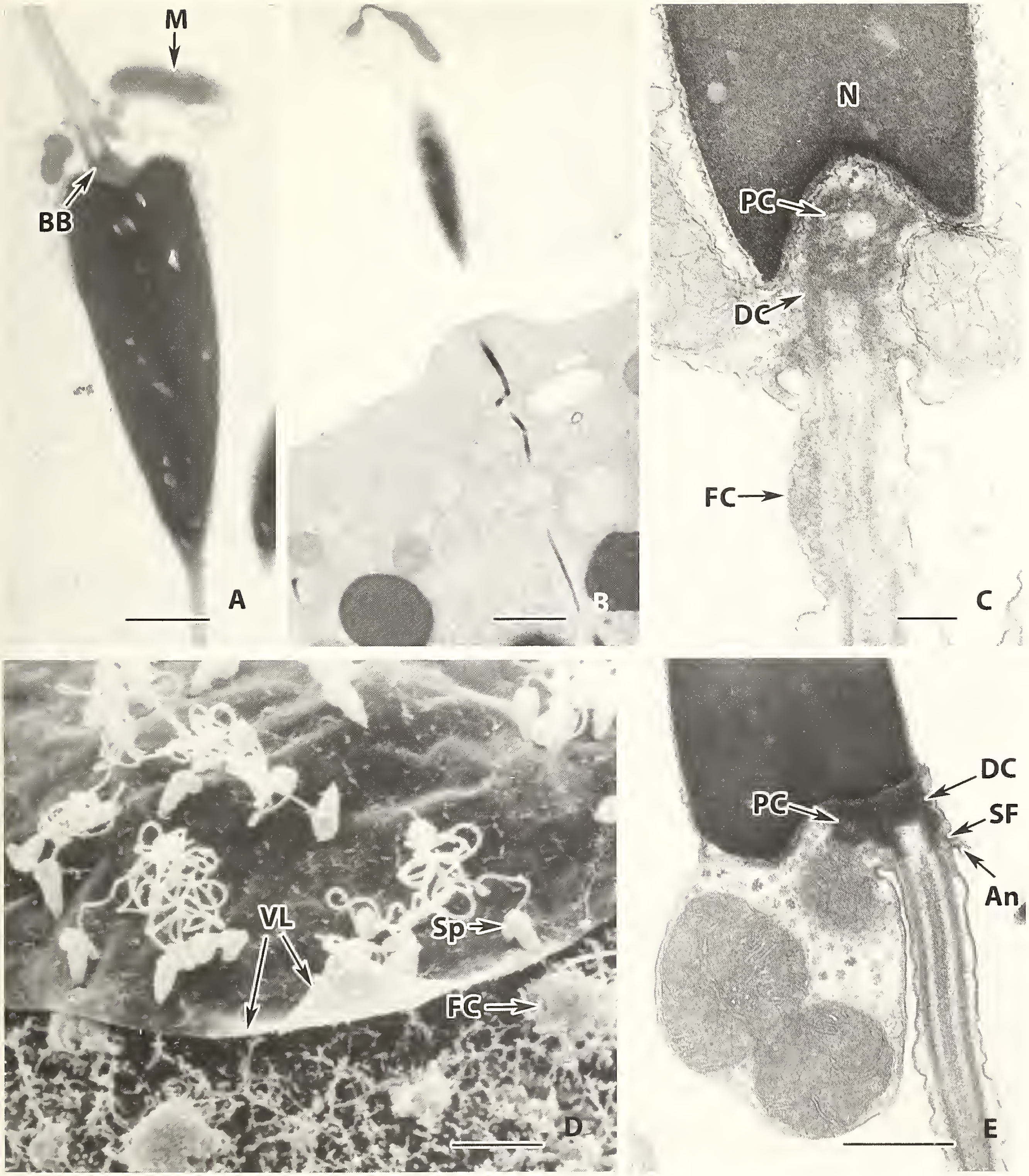


Figure 3. A, Quarter of egg of *Leptochiton asellus* viewed with SEM, showing smooth surface of coat without pores. Scale bar = 25 µm. B, Quarter of egg of *Callochiton dentatus* viewed with SEM, showing regularly spaced pores in smooth jelly coat. Scale bar = 25 µm. C, Quarter of egg of *Stenosemus albus* viewed with SEM, showing simple spines with hooked tips and hexagonal bases measuring 36 µm across. Scale bar = 50 µm. D, Quarter of egg of the brooding chiton *Radsia nigrovirescens* showing long simple spines with hooks which interlock in the pallial grooves retaining eggs. Scale bar = 50 µm.

→

Figure 4. A, TEM of sperm of *Callochiton dentatus* showing oblong mitochondrion (M) and centriolar basal body (BB) acentric to nucleus. Scale bar = 1 µm. B, TEM of sperm penetrating egg of *C. dentatus*, showing long thread of chromatin in egg cortex. Pore in vitelline layer is too small to admit sperm organelles which, like the elongate mitochondrion, appear to be abandoned on the surface in a bag of membrane. Scale bar = 1 µm. C, TEM of sperm of *Mopalia muscosa* showing proximal centriole (PC) fused to apex of distal centriole (DC) in fossa of nucleus (N). Flagellum is reinforced by a fibrous complex (FC), which characterizes the suborder Acanthochitonina. Scale bar = 0.2 µm. D, SEM of polyspermic fertilization of *Callochiton dentatus* egg, showing multiple sperm (Sp) penetrating vitelline layer (VL)



and the induction of several fertilization cones (FC). Note: jelly layer has been completely dissolved. Scale bar = 5 µm. E, TEM of sperm of *Chaetopleura apiculata*, showing basal body comprised of proximal centriole (PC) fused laterally to distal centriole (DC), which produces an acentric flagellum. Thickening of membrane is visible extending posteriorly from annulus (An) on both sides. Mitochondria with glycogen granules are arranged in posterior extension of mid-piece. Scale bar = 0.5 µm.

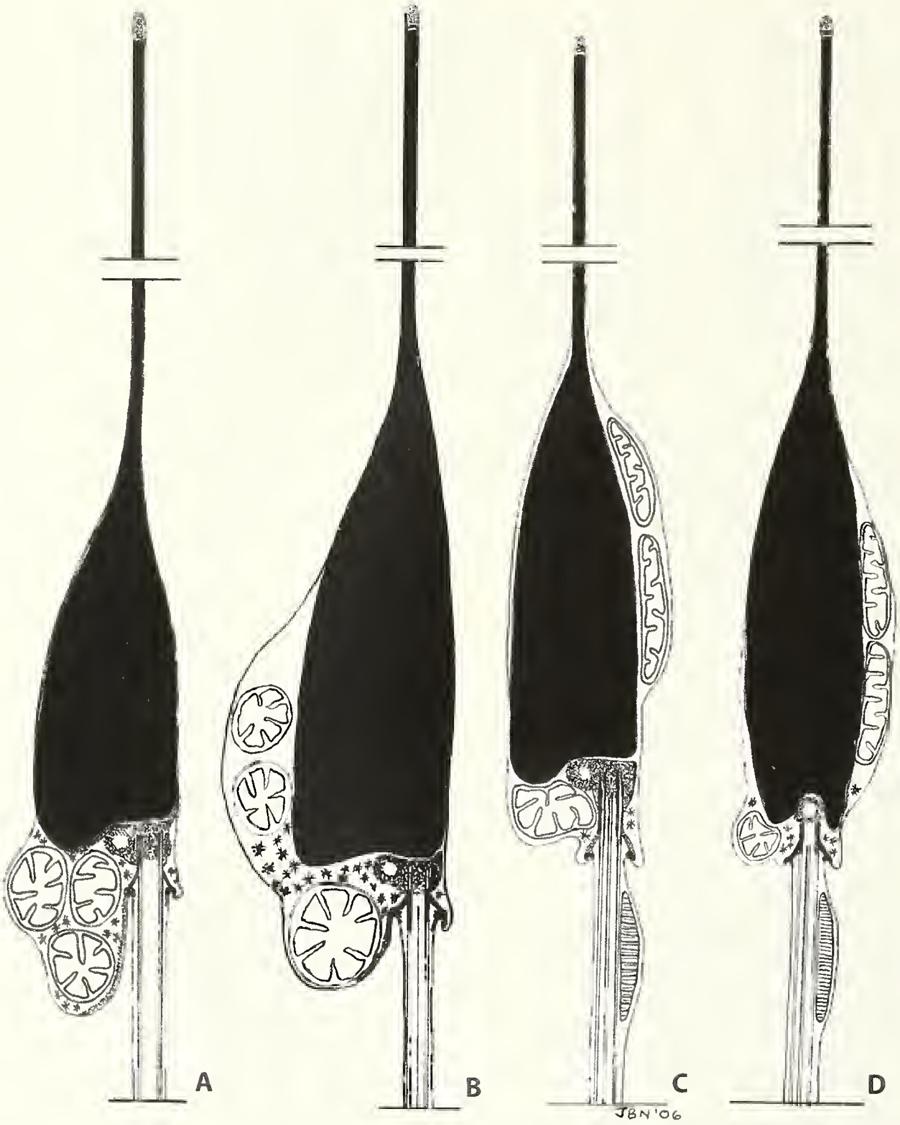


Figure 5. A, Sperm of *Chaetopleura apiculata*; B, sperm of *Stenoplax conspicua*; C, Sperm of *Acanthochitona viridis*; and D, sperm of *Mopalia muscosa*.

on the side opposite to the mitochondria (Figs. 4C, 5C-D). More specific differences in the arrangement of centrioles in the basal body and in the structure of the acrosome may distinguish among two or more groups in this suborder. For example, in *Cyanoplax* and *Acanthochitona* the proximal centriole fuses laterally with the distal centriole towards the axis of the sperm, as was found in Chitonina (Fig. 5C). However, in the genera *Tonicella* Carpenter, 1873, *Cryptochiton* Middendorff, 1847, and *Mopalia* Gray, 1847, the proximal centriole fuses to the anterior of the distal centriole and is embedded in a small nuclear fossa (Figs. 4C, 5D).

Acanthochitonina species also are united by having elaborate egg hulls raised into large cupules with wide bases ranging in size from 50 to 90 μm (Figs. 8A-D). Two main types of fertilization have been observed which are defined also by cupule morphology. The first involves closed-hull cupules with small pores in the intercupule area that give sperm direct access to the vitelline layer (Fig. 7F). The second type involves open-hull cupules with sperm swimming inside and penetrating both hull and vitelline layer without access to any pores (Fig. 7G). In both cases, the sperm inject the chromatin into the egg through the narrow nuclear filament. Other sperm organelles, including mitochondria and centrioles, appear to be excluded and left behind on the egg surface in a bag of sperm membrane (Fig. 7C).

Some species with closed cupules are brooders and in some of these, such as *Cyanoplax fernaldi* (= *Lepidochitona fernaldi* Eernisse, 1986) (Fig. 8C), the cupules are reduced but maintain the same form as non-brooding species, such as *Cyanoplax dentiens* (= *Lepidochitona dentiens* (Gould, 1846)) (Fig. 8C). Among open-cupule species some have a more complex, folded-cupule structure with protruding elements inside each cupule (Fig. 7G) (*e.g.*, the genera *Tonicella*, *Cryptochiton*, and *Mopalia* of those studied here). However, *Nuttallina californica* (Nuttall MS, Reeve, 1847) and related species do not have these protruding structures (Fig. 8D).

Cladistic analysis

Sperm and egg characters for all taxa are summarized (Tables 2-3). The data matrix (Table 2) was run through branch and bound analysis in PAUP 4.0. The Bootstrap consensus tree (for 500 replicates) resulting from this analysis is shown with confidence values written above the line for each node (Fig. 9). Lepidopleurida came out as paraphyletic in this consensus tree, largely because characters for egg and sperm shared by *Deshayesiella curvata* and *Hanleya hanleyi* were coded as apomorphic (Table 4). There was 100% support for a monophyletic Chitonida, which includes Callochitonidae as the sister taxon to Chitonina plus Acanthochi-

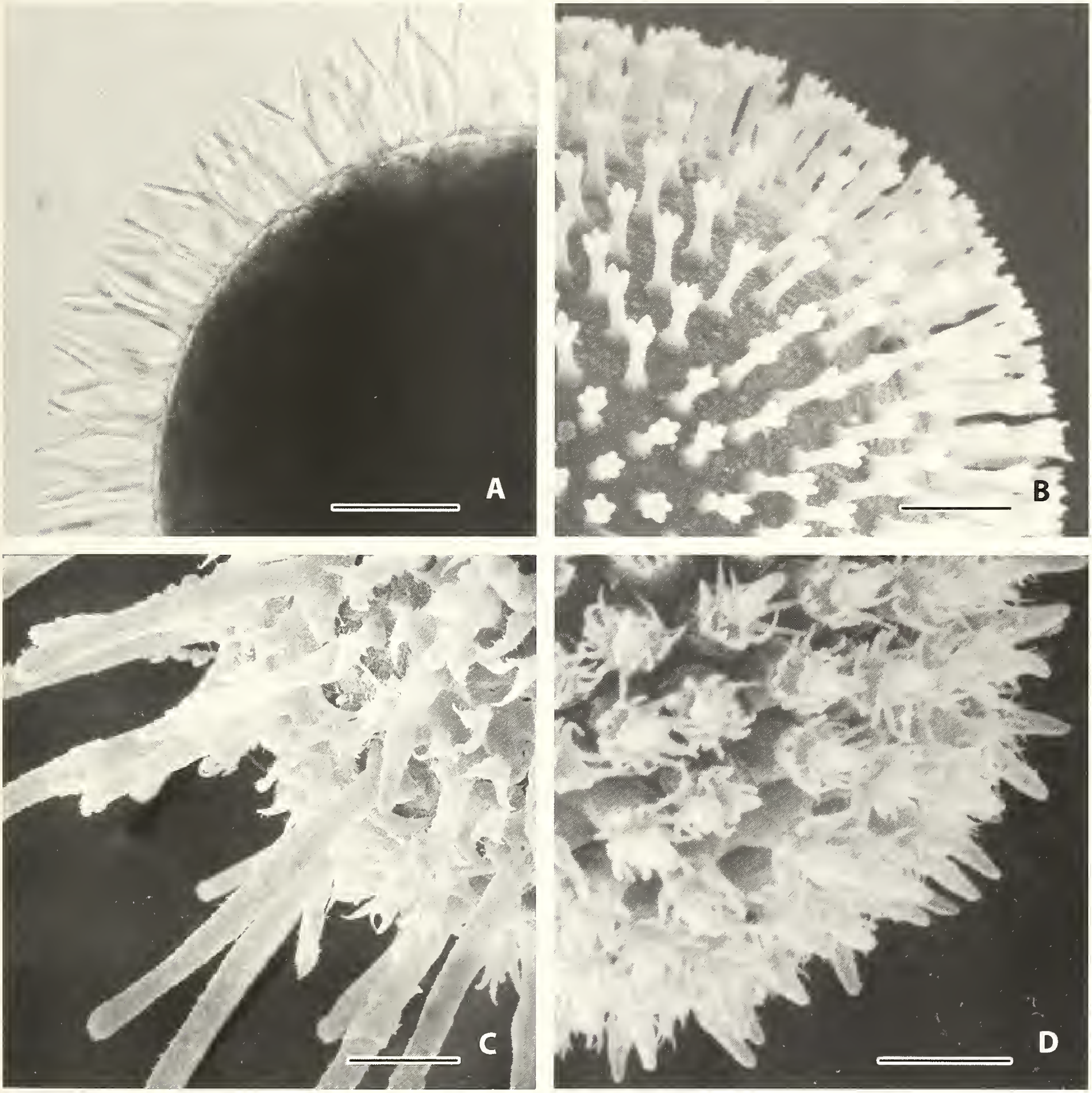


Figure 6. A, Quarter of egg of *Stenoplax conspicua* viewed with DIC optics, showing spines with bifurcating tips, which characterize this and other genera, including *Lepidozona*. Scale bar = 25 μm. B, Quarter of egg of *Rhyssoplax tulipa* viewed with SEM, showing spines with petalloid tips, typical of this genus and of some species of *Ischnochiton*. Scale bar = 20 μm. C, Quarter of egg of *Acanthopleura granulata* viewed with SEM, showing unique polymorphic spines. Some spines are elongate with a scale-like outer layer; others are intermediate in length and a third type is short and bifurcates at the tip. Scale bar = 20 μm. D, Quarter of egg of *Chaetopleura apiculata* viewed with SEM, showing complex branching spines that may be unique to this genus. Scale bar = 30 μm.

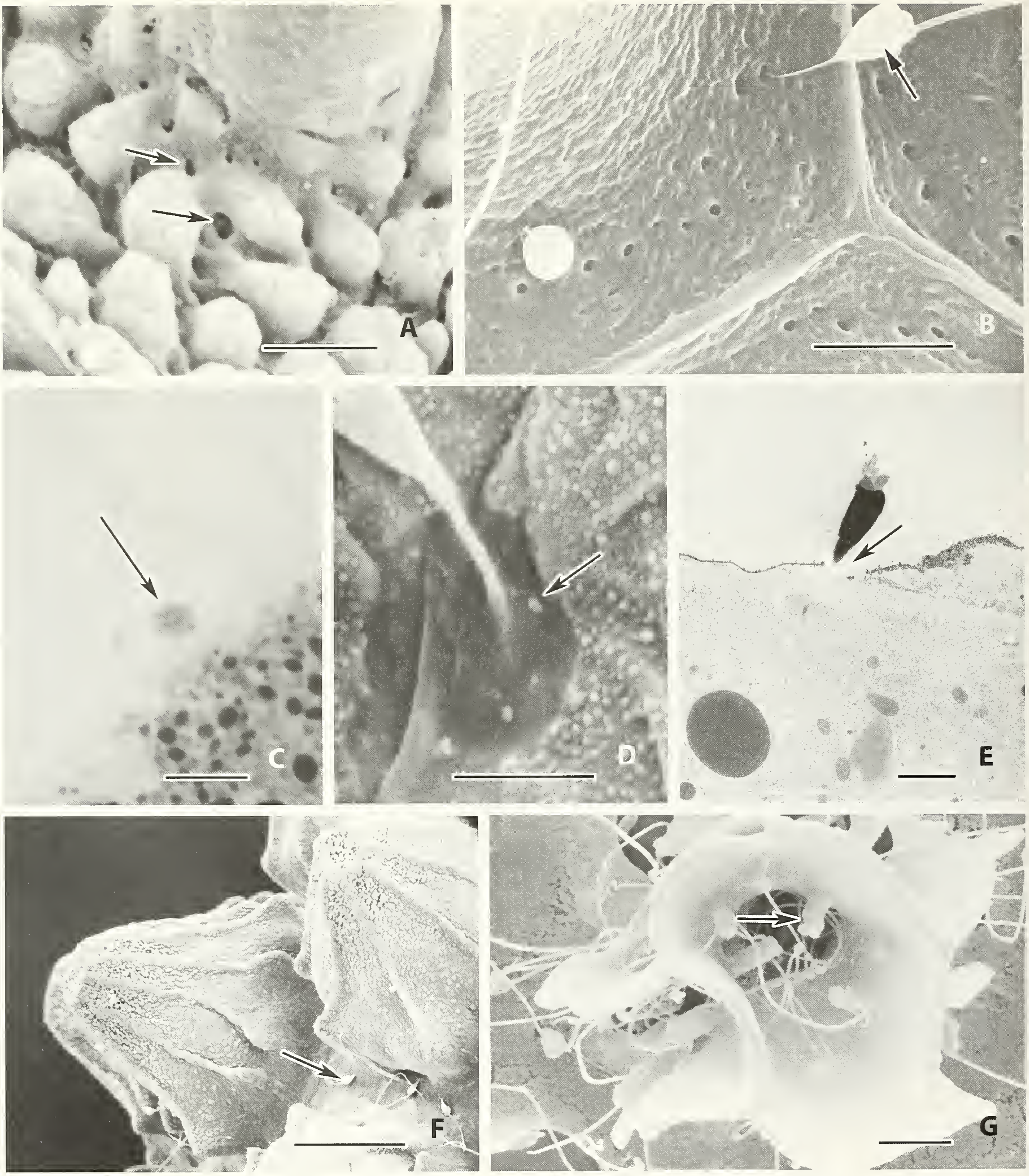


Figure 7. A, SEM detail at base of spine on egg of *Radsia nigrovirescens* showing pores in hull (arrows). Scale bar = 3 µm. B, SEM of egg of *Stenosemus albus* showing pores at perimeter of hexagonal bases of spines, which allow sperm (arrow) direct access to vitelline layer. Scale bar = 1 µm. C, LM of 1 µm section of *Mopalia muscosa* egg fertilized two hours before with dilute sperm suspension. Serial sections revealed only one membrane bag containing eight or nine particles which correspond to the roughly eight mitochondria and centrioles of this sperm (Buckland-Nicks and Brothers, unpubl. micrograph). Scale bar = 1 µm. D, SEM of sperm penetrating egg of *Stenoplax conspicua* showing

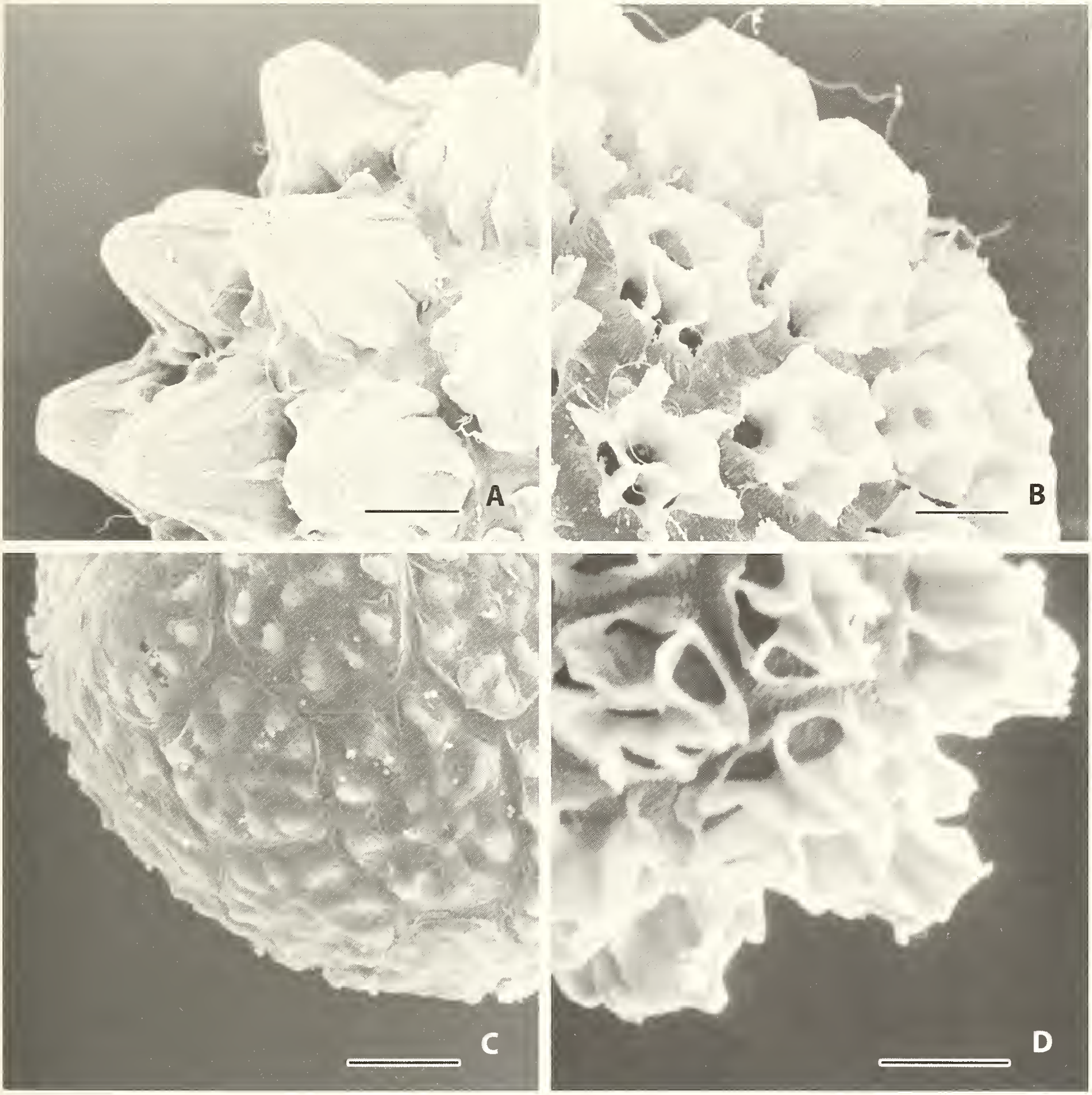


Figure 8. A, Quarter of egg of *Cyanoplax dentiens* viewed with SEM, showing closed hull cupules typical of this genus, as well as *Acanthochitona*. Scale bar = 25 µm. B, Quarter of egg of *Mopalia muscosa* viewed with SEM, showing open cupules typical of this clade. Scale bar = 25 µm. C, Quarter of egg of brooding chiton *Cyanoplax fernaldi* viewed with SEM, showing reduced hull cupules with typical hexagonal bases. Micrograph courtesy of D. Eernisse (see also Eernisse 1984). Scale bar = 30 µm. D, Quarter of egg of *Nuttallina californica* viewed with SEM, showing the type of open-hull cupules that lack internal protrusions. Scale bar = 20 µm.

←

dissolution of outer dense layer around nuclear filament (arrow). Scale bar = 1 µm. E, TEM of sperm penetrating egg of *Rhyssoplax tulipa* showing dissolution of outer dense layer around nuclear filament (arrow). Scale bar = 2 µm. F, Fertilized egg of *Cyanoplax dentiens* showing penetrating sperm (arrow) between closed cupules. Scale bar = 10 µm. G, Fertilized egg of *Mopalia muscosa* showing protrusions (arrow) from inside wall of hull cupules that are absent from cupules of *Nuttallina californica*. Scale bar = 5 µm.

Table 2. Characters used in cladistic analysis of Polyplacophora and Aplacophora (outgroups) with character state codes in boldface font (see Table 3 for data matrix).

SPERM AND EGG DATA

(1) Acrosome: **0:** Acrosome forms from Golgi body in posterior of spermatid; **1:** Acrosome forms by aggregation of small proacrosome vesicles at filament tip.
(2) Acrosome structure: **0:** Cone with subacrosomal granule (SAG), interstitial granule (IG), and subacrosomal plate (SAP); **1:** Cone with SAG and SAP; **2:** Vesicle with SAP.
(3) Mitochondria position: **0:** Sheath around flagellum; **1:** Ring around centrioles; **2:** Ring around offset basal body; **3:** Basal mitochondria in collar; **4:** Lateral mitochondria; **5:** Lateral and anterior mitochondria.
(4) Mitochondria number: **0:** 1-2; **1:** 3-4; **2:** 5-6; **3:** 7-9.
(5) Mitochondrial shape: **0:** Fused spiral; **1:** All spherical; **2:** Not all spherical.
(6) Nuclear filament: **0:** Absent; **1:** 1-2 μm; **2:** ≥3 μm.
(7) Flagellum reinforcement: **0:** Absent; **1:** Spiral ribbon; **2:** Thickened membrane; **3:** Unilateral fibrous body.
(8) Chromatin pattern: **0:** Granular; **1:** Thick short fibers; **2:** Fine then coarse fibers; **3:** Fine long fibers.
(9) Centrioles: **0:** Basal body in deep nuclear fossa; **1:** Separate centrioles; **2:** Proximal centriole (PC) fused lateral to distal centriole (DC) and offset; **3:** PC fused anterior to DC in nuclear fossa.
(10) Hull projections: **0:** Absent; **1:** Spines with bases 5-30 μm; **2:** Cupules with bases 50-90 μm.
(11) Hull cupules: **0:** Absent; **1:** Closed; **2:** Open.
(12) Jelly coat: **0:** Absent; **1:** Smooth without pores; **2:** Smooth with large pores >5 μm.
(13) Hull structure: **0:** Jelly coat; **1:** Jelly coat with macropores; **2:** Hull with micropores in spines; **3:** Hull with dense layer; **4:** Hull with micropores between cupules; **5:** Cupules without pores.
(14) Fertilization site: **0:** Internal **1:** External: anywhere on egg; **2:** Sperm enters specific site.
(15) Site-specific sperm entry: **0:** Absent; **1:** Macropores >5 m; **2:** Between hull projections; **3:** Inside hull projections.
(16) Fertilization cone: **0:** Engulfs sperm and organelles; **1:** Engulfs only chromatin.

OTHER MORPHOLOGICAL DATA

(17) Gill position: **0:** Reduced; **1:** Adanal; **2:** Abanal.
(18) Gill type: **0:** Absent; **1:** Merobranchial; **2:** Holobranchial.
(19) Insertion plate: **0:** Absent; **1:** Present; **2:** Slitted; **3:** Pectinated.
(20) Body covering: **0:** Spinous; **1:** 8 shell valves.
(21) Foot: **0:** Absent; **1:** Present.
(22) Shell valves: **0:** Absent; **1:** Modern valve shape; **2:** Terminal valves with fissures.

tonina. There was also strong support (96%) for monophyly of the rest of Chitonida (= Chitonina + Acanthochitonina). However, there was weaker support (65%) for a monophyletic Chitonina, even though a number of apomorphic characters are shared by this grouping (Table 4). Also, there was similar support (62%) for a monophyletic grouping of Acanthochitonina, members of which share a different set of apomorphic characters (Table 4). However, there was strong support (97%) for a monophyletic Mopaliidae, which included *Mopalia*, *Cryptochiton*, and *Tonicella*. The apomorphy hypotheses for each of 8 selected internal nodes are listed in Table 4.

DISCUSSION

Mechanisms of fertilization

Lepidopleurida: Leptochitonidae

Confirmation of the extrusion of an acrosomal process (Buckland-Nicks 2006) and breaching of the vitelline layer by the sperm acrosome indicates that *Leptochiton asellus* penetrates the egg in a manner similar to that of most other Metazoa, including scaphopods (Dufresne-Dubé *et al.* 1983) and sea urchins (Longo 1987) although this remains to be confirmed. Other Lepidopleurida, such as *Deshayesiella curvata* and *Hanleya hanleyi*, are likely to do the same as extrusion of an acrosomal process has been observed in *D. curvata* (Buckland-Nicks, unpubl. data), and sperm of *H. hanleyi* are constructed in the same way. However, this will require confirmation by direct observation of sperm organelles following fertilization. The current analysis showed Lepidopleurida to be paraphyletic in agreement with Eernisse *et al.* (2006). However, this result depends largely on coding changes in sperm and egg structure in *Deshayesiella* and *Hanleya* as derived, rather than evolving by convergence. Recent molecular analyses (Eernisse, unpubl. data) have shown that these genera form part of a monophyletic Leptochitonidae. A combined molecular and morphological analysis would produce the most robust test of these phylogenetic relationships but for the moment we will continue to regard Lepidopleurida as the basal monophyletic order, as in Sirenko (2006).

Callochiton dentatus, although retaining the plesiomor-

Table 3. Data matrix showing characters and their character states for the species listed. A, Acrosome; As, Acrosome structure; Mp, Mitochondria position; M#, Mitochondria number; M, Mitochondria shape; Nf, Nuclear filament; Fr, Flagellum reinforcement; Ch, Chromatin pattern; Ce, Centrioles; Hp, Hull projections; Hc, Hull cupules; Jc, Jelly coat; Hs, Hull structure; Fs, Fertilization site; Se, Site specific sperm entry; Fc, Fertilization cone; Gp, Gill position; Gt, Gill type; Ip, Insertion plates; Bc, Body covering; F, Foot; Sv, Shell valves; ?, unknown state; *, outgroups.

Character type →	Ac	As	Mp	M#	M	Nf	Fr	Ch	Ce	Hp	Hc	Jc	Hs	Fs	Se	Fc	Gp	Gt	Ip	Bc	F	Sv
↓Genus/species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
*Epimenia australis**	0	0	0	0	0	0	1	?	0	0	0	1	01	0	0	?	0	0	0	0	0	0
*Chaetoderma argenteum**	0	2	1	2	1	0	0	0	1	0	0	1	0	1	0	?	0	0	0	0	0	0
Leptochiton asellus	0	1	1	2	1	0	0	1	1	0	0	1	0	1	0	0	1	1	0	1	1	1
Deshayesiella curvata	0	1	1	2	1	1	0	1	1	0	0	1	1	2	1	0	1	1	0	1	1	1
Hanleya hanleyi	0	1	1	2	1	1	0	1	1	0	0	1	1	2	1	0	1	1	1	1	1	1
Callochiton dentatus	1	2	2	2	2	2	0	2	2	0	0	1	1	2	1	1	1	1	2	1	1	1
Chaetopleura apiculata	1	2	3	1	2	2	2	2	2	1	0	0	2	2	2	1	1	2	2	1	1	2
Stenosemus albus	1	2	3	1	2	2	2	2	2	1	0	0	2	2	2	1	1	2	2	1	1	2
Acanthopleura granulata	1	2	3	1	2	2	2	2	2	1	0	0	2	2	2	1	1	2	3	1	1	2
Rhyssoplax tulipa	1	2	3	1	2	2	2	2	2	1	0	0	2	2	2	1	1	2	3	1	1	2
Ischnochiton hakodadensis	1	2	4	1	2	2	2	2	2	1	0	0	2	2	2	1	1	2	2	1	1	2
Stenoplax conspicua	1	2	4	1	2	2	2	2	2	1	0	0	2	2	2	1	1	2	2	1	1	2
Lepidozona retiporosa	1	2	4	1	2	2	2	2	2	1	0	0	2	2	2	1	1	2	2	1	1	2
Cyanoplax dentiens	1	2	5	3	2	2	3	3	2	2	1	0	3	2	2	1	2	1	2	1	1	2
Acanthochitona viridis	1	2	5	3	2	2	3	3	2	2	1	0	3	2	2	1	2	1	2	1	1	2
Tonicella lineata	1	2	5	3	2	2	3	3	3	2	2	0	4	2	3	1	2	2	2	1	1	2
Cryptochiton stelleri	1	2	5	3	2	2	3	3	3	2	2	0	4	2	3	1	2	2	2	1	1	2
Mopalia muscosa	1	2	5	3	2	2	3	3	3	2	2	0	4	2	3	1	2	2	2	1	1	2

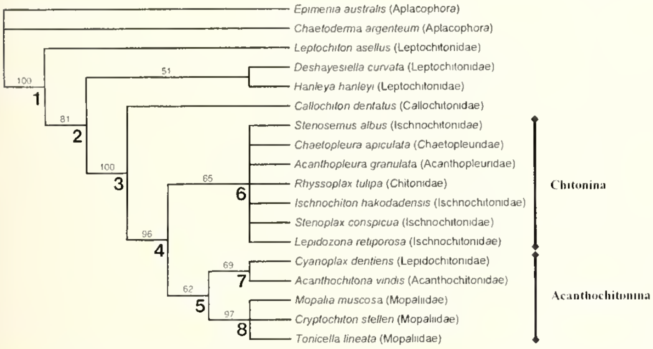

Epimenia australis (Aplacophora)
Chaetoderma argenteum (Aplacophora)
Leptochiton asellus (Leptochitonidae)
Deshayesiella curvata (Leptochitonidae)
Hanleya hanleyi (Leptochitonidae)
Callochiton dentatus (Callochitonidae)
Stenosemus albus (Ischnochitonidae)
Chaetopleura apiculata (Chaetopleuridae)
Acanthopleura granulata (Acanthopleuridae)
Rhyssoplax tulipa (Chitonidae)
Ischnochiton hakodadensis (Ischnochitonidae)
Stenoplax conspicua (Ischnochitonidae)
Lepidozona retiporosa (Ischnochitonidae)
Cyanoplax dentiens (Lepidochitonidae)
Acanthochitona viridis (Acanthochitonidae)
Mopalia muscosa (Mopaliidae)
Cryptochiton stelleri (Mopaliidae)
Tonicella lineata (Mopaliidae)
Chitonina
Acanthochitonina
100
1
81
2
51
100
3
96
4
65
6
69
7
62
5
97
8

Figure 9. Consensus tree from branch and bound analysis in PAUP 4.0 of the data matrix in Table 2. Numbers above nodes represent the bootstrap values for 500 replicates. Numbers below refer to internal nodes. Apomorphy hypotheses describing these nodes are listed in Table 4.

Table 4. Apomorphy hypotheses for selected internal nodes of bootstrap consensus tree shown in Fig. 9. Unless otherwise stated in brackets, character changes are from 0 →1.

Internal node	Character changes
Node 1: Polyplacophora	17, 18, 20, 21, 22
Node 2: *Deshayesiella* + *Hanleya*	6
Node 3: Chitonida	1, 2 (1-2), 3 (1-2), 5 (1-2), 6 (1-2), 8 (1-2), 9(1-2), 16, 19 (1-2)
Node 4: Chitonina + Acanthochitonina	3 (2-3), 4 (2-1), 10, 12 (1-0), 15 (1-2), 18 (1-2), 22 (1-2)
Node 5: Acanthochitonina	3 (4-5), 4 (1-3), 7 (2-3), 8 (2-3), 10 (1-2), 11, 13 (2-3), 17 (1-2)
Node 6: Chitonina	7 (0-2), 13 (1-2)
Node 7: *Cyanoplax* + *Acanthochitona*	11, 13 (3-4), 18 (2-1)
Node 8: *Mopalia* + *Cryptochiton* + *Tonicella*	9 (2-3), 11 (1-2), 13 (4-5), 15 (2-3)

phic egg type first discovered in *Deshayesiella curvata* (Pashchenko and Drozdov 1998), has evolved several apomorphic features in the sperm, which dictate a novel mechanism of fertilization, and one that characterizes the order Chitonida (Buckland-Nicks and Hodgson 2000, Buckland-Nicks 2006). In this mechanism a permanent nuclear filament has replaced the extrusion of an acrosomal process; after the tiny acrosome digests a small hole in the vitelline layer, the nuclear filament fuses with an egg microvillus and delivers the chromatin into the egg. Furthermore, current evidence suggests that other sperm organelles, including mitochondria and centrioles, do not pass through this minute tube and are left behind on the surface (Buckland-Nicks and Hodgson 2000, Buckland-Nicks 2006, Buckland-Nicks and Brothers 2008). These characteristics place Callochitonidae firmly within Chitonida and outside the Lepidopleurida

(Fig. 9). This result is different than that obtained by one molecular analysis (Okusu *et al.* 2003) but agrees with previous results based on morphological data (Buckland-Nicks 1995, 2006, Sirenko 1993, 2006) or DNA sequences encoding for hemocyanin protein (Lieb *et al.* 2006).

Chitonida: Chitonina

The suborder Chitonina is unified by synapomorphic gamete characters (Table 4). In the sperm, these include the very acentric basal bodies with proximal centrioles positioned perpendicular and lateral to the distal centriole in a fused mass towards the mitochondrial side (see also Hodgson *et al.* 1988) and a dense thickening that extends from the annulus along the plasma membrane adjacent to the mitochondria (Buckland-Nicks 2006). In the egg, synapomorphic characters include hull spines with narrow bases (Sirenko 1993, 2006, Buckland-Nicks 2006). New data on fertilization in *Chaetopleura apiculata* (Buckland-Nicks and Brothers 2008) and *Stenosemus albus* indicate that they and their close relatives are more basal among Chitonina, a result which was found also by Okusu *et al.* (2003) in their combined molecular analysis (p. 293, fig. 6) and combined morphological analysis (p. 295, fig. 8). However, the consensus tree in the present study found weak support (65%) for a monophyletic Chitonina and was unable to distinguish between the families in this suborder. A combined molecular and morphological analysis would likely provide a more robust test with better resolution.

This study supports the finding of Okusu *et al.* (2003) that the genus *Ischnochiton* is polyphyletic. Okusu *et al.* (2003) showed that *Ischnochiton rissoi* (Payraudeau, 1826) is closely related to Chaetopleurinae whereas other *Ischnochiton* species came out within Ischnochitoninae. Spine form in general seems to be a good indicator of relationship among genera such as *Stenoplax* and *Lepidozona* (bifurcating spines) as well as *Chiton* Linnaeus, 1758 and *Rhyssoplax* Thiele, 1893 (petalloid spines), whereas the mosaic of spine form found among different species of *Ischnochiton* suggests polyphyly (see diagrams by Sirenko 1993 and 2006). The present study supports reclassification of *Ischnochiton albus* (Linnaeus, 1767) as *Stenosemus albus* (see Sirenko 2006). Only further studies on the reproductive biology and molecular biology of other species of *Ischnochiton* will resolve any further inconsistencies in classification.

Radsia nigrovirescens is an anomaly among Chitoninae as it has pores in the hull and an unusual spine form, more like that of *Stenosemus albus.* Furthermore, it is a brooder and in most brooders the spines are reduced or absent (Eernisse 1984, Buckland-Nicks and Eernisse 1992). Fertilization biology has not yet been studied and it remains unknown if the sperm penetrate these pores, as occurs in *Stenosemus* and *Chaetopleura*, or digest the egg hull to make an open pathway, as in other Chitoninae (Buckland-Nicks 2006).

Acanthopleura granulata is unique among chitons examined to date, as it has evolved polymorphic spines, which include long and intermediate scaly ones, as well as short bifurcating ones. Previously, eggs of *A. granulata* were illustrated with straight spines ending in points (Sirenko 1993, 2006), which is clearly a simplification. A recent analysis of a related species, *Acanthopleura echinatus* Barnes, 1824 (Gaymer *et al.* 2004), revealed spines with petalloid tips, like some other genera of Chitoninae, including *Chiton* and *Rhyssoplax*. In keeping with this, the hull does not have pores like *Chaetopleura* and *Stenosemus*, suggesting that the mechanism of fertilization is more like that in genera of Chitoninae, such as *Chiton*, *Rhyssoplax*, or of Ischnochitoninae, such as *Stenoplax* and *Lepidozona*. However, a recent molecular analysis (Eernisse, unpubl. data) indicates that Acanthopleurinae + Toniciinae is separate from Chitoninae.

Chitonida: Acanthochitonina

Members of the suborder Acanthochitonina share synapomorphies for sperm, with a fibrous complex on the flagellum and anterior mitochondria, and for eggs, with broad based cupules. Also this suborder is characterized by abanal gills (Sirenko 1993, 2006). However, two groups emerge within this suborder, one united by closed cupules, laterally fused centrioles and fertilization between cupules (*e.g.*, *Cyanoplax* Pilsbry, 1892 and *Acanthochitona* Gray, 1821), and the second united by open cupules, anteriorly fused centrioles, and fertilization inside cupules (*Mopalia*, *Tonicella*, *Cryptochiton*) (see Table 4). *Cyanoplax cinerea* (= *Lepidochitona cinerea* Linnaeus, 1767) may represent an exception to this, as it is reported to have open cupules (Eernisse 1984). In the present analysis, *Cyanoplax* places outside Mopaliidae, as was reported by Buckland-Nicks (1995) and Okusu *et al.* (2003: figs. 6, 8). This disagrees with Sirenko (2006), who placed this genus within Tonicellidae. Furthermore, in this analysis *Cryptochiton* comes out with *Mopalia* and *Tonicella* within Mopaliidae, a placement which agrees with Okusu *et al.* (2003: figs. 6, 8) and Eernisse (pers. comm.). However, Sirenko (2006) placed *Cryptochiton* in Acanthochitonidae. Okusu *et al.* (2003: fig. 7B) suggested that there is a possibility that Acanthochitonina species are paraphyletic based on one of two consensus trees for combined molecular data. However, based on morphology alone for the genera included here, this study finds some support (62%) for a monophyletic Acanthochitonina, as was also shown by the second consensus tree of Okusu *et al.* (fig. 7A: 2003).

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Chitons (Mollusca: Polyplacophora) associated with hydrothermal vents and methane seeps around Japan, with descriptions of three new species*

Hiroshi Saito[1], Katsunori Fujikura[2], and Shinji Tsuchida[3]

[1] Department of Zoology, National Museum of Nature and Science, Tokyo, 3-23-1 Hyakunin-cho, Shinjuku-ku, Tokyo 169-0073 Japan, h-saito@kahaku.go.jp

[2] Japan Agency for Marine-Earth Science and Technology, 2-15 Natsushima, Yokosuka-shi, Kanagawa 237-0061 Japan, fujikura@jamstec.go.jp

[3] Japan Agency for Marine-Earth Science and Technology, 2-15 Natsushima, Yokosuka-shi, Kanagawa 237-0061 Japan, tsuchidas@jamstec.go.jp

Abstract: Three new species of chitons are described from hydrothermal vent sites and methane seep sites around Japan: ***Deshayesiella sirenkoi*** **n. sp.** from the hydrothermal vent sites on the seamounts in the northern Mariana Islands area, ***Placiphorella okutanii*** **n. sp.** from Hachijo Depression in the Izu-Ogasawara (Bonin) Islands area where no active vent/seep area has been discovered, but the possibility of hydrothermal activity has been suggested, and ***Placiphorella isaotakii*** **n. sp.** from methane seep sites on the Kuroshima Knoll off Yaeyama Islands. ***Deshayesiella sirenkoi*** **n. sp.** as well as two previously known hydrothermal vent species, *Leptochiton tenuidontus* Saito and Okutani, 1990 and *Thermochiton undocostatus* Saito and Okutani, 1990, are vent/seep associated species, whereas the two *Placiphorella* may be guest species. Additional distributional records are given for the two known species.

Key words: *Deshayesiella*, *Placiphorella*, deep-sea, chemosynthetic environment, taxonomy, Pacific Ocean

The number of the molluscan taxa described from chemosynthetic environments has rapidly increased in the last two decades (Sasaki *et al.* 2005). Most of these taxa are, however, gastropods and bivalves (Desbruyères *et al.* 2006: 520-523). Since Saito and Okutani (1990) described two chiton species from the hydrothermal vent site of Okinawa Trough, East China Sea, some chiton species have been reported from chemosynthetic environments. Squires and Goedert (1995) reported *Leptochiton alveolus* (Lovén, 1846) (*sensu* Ferreira 1979 and Kaas and Van Belle 1985) from Eocene and Oligocene cold methane seep limestones, Olympic Peninsula, Washington. Olu *et al.* (1997) reported "Polyplacophora" from the methane seep of Barbados Prism, 1,000-2,000 m, and Sellanes *et al.* (2004) reported *Leptochiton* sp., *Stenosemus* sp., and *Placiphorella* sp. from methane seepage in the bathyal zone off Chile. Schwabe and Sellanes (2004) have described a new species, *Lepidozona balenophila*, from another type of chemosynthetic environment, decomposing whale carcasses. However, no vent/seep associated chiton species, other than the two known species, has been described anywhere else in the world. Those two known species that were described from the hydrothermal vent in the East China Sea are *Leptochiton tenuidontus* Saito and Okutani, 1990 and *Thermochiton undocostatus* Saito and Okutani, 1990. They were collected by a human-occupied submersible, *Shinkai 2000*, belonging to Japan Agency for Marine-Earth Science and Technology (JAMSTEC). Since then, some additional chiton specimens have been collected from Japanese waters by the Deep Sea Research System, including human-occupied submersibles or ROVs belonging to JAMSTEC. Here, we describe three new species and further describe the morphology and distribution of the two previously known species.

MATERIALS AND METHODS

Specimens were collected by the Deep-sea Research System of JAMSTEC: human-occupied submersibles *Shinkai 2000* (abbreviated as 2K) and *Shinkai 6500* (6K) and a remotely operated vehicle *Hyper-Dolphin* (HPD). Sampling sites are shown in Fig. 1. Preparation for SEM observation followed Saito (2006). All specimens were deposited in the molluscan collection of the Department of Zoology, National Museum of Nature and Science (formerly National Science Museum, Tokyo) (NSMT).

The systematic arrangement used in this paper follows Sirenko (2006).

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SYSTEMATICS

Order Lepidopleurida Thiele, 1909
Suborder Lepidopleurina Thiele, 1909
Family Leptochitonidae Dall, 1889
Genus *Leptochiton* Gray, 1847

Type species

Chiton cinereus Montagu, 1803 [= *Leptochiton asellus* (Gmelin, 1791)], by subsequent designation (Gray, 1847).

Leptochiton tenuidontus Saito and Okutani, 1990
(Fig. 2A-B)

Leptochiton tenuidontus Saito and Okutani 1990: 166-171, figs. 2-12, pl. 1, figs. 1-4; Kaas and Van Belle 1994: 22-23, fig. 7; Kaas and Van Belle 1998: 185; Saito 2000: 7, pl. 3, fig. 11; Cosel 2006: 81.
Leptochiton sp. Saito and Fujikura 2000: 74-75.

Type material examined

Holotype: NSMT-Mo 69193, body length ca. 16 mm. Type locality: hydrothermal vent site on the Iheya Ridge, central Okinawa Trough, East China Sea, 27°32.70′N, 126°58.20′E, 1395 m, 2K, Dive #426, 21 July 1989.

Additional material examined

NSMT-Mo 73838 (ex. JAMSTEC sample No.: RK4-A-1, 009550-009553), 4 specimens, body length ca. 18-20 mm, methane seep site off Kikaijima Island in the Amami Islands area, 28°26.39′N, 130°19.01′E, 1430 m, 2K, Dive #1020, 24 June 1998; NSMT-Mo 73839 (ex. JAMSTEC sample No.: RK4-A-1, 009548-009549), 2 specimens, body length 22 and 23 mm, methane seep site off Kikaijima Island in the Amami Islands area, 28°26.42′N, 130°18.98′E, 1442 m, 2K, Dive #1021, 25 June 1998; NSMT-Mo 73940 (ex. JAMSTEC sample No.: RK4-A-1, 009544-009547), 3 specimens, body length ca. 20-22 mm, methane seep site off Kikaijima Island, in the Amami Islands area, 28°26.45′N, 130°19.1′E, 2K, Dive #1022, 1440 m, 26 June 1998. All nine specimens were found on the shells of *Bathymodiolus platifrons* Hashimoto and Okutani, 1994.

Additional description

Tegmentum sculptured with round granules densely arranged in quincunx order on head valve, lateral areas of median valves, and postmucronal area of tail valve, with elongate granules arranged in quincunx order or, occasionally, in irregular longitudinal rows in central area of median valves and antemucronal area of tail valve (Fig. 2A). Each granule with one macraesthete pore and one to four micraesthete pores on anterior slope; size of macraesthete pore ca. 5-8 μm, that of micraesthete pore slightly smaller than macraesthete pore (Fig. 2B).

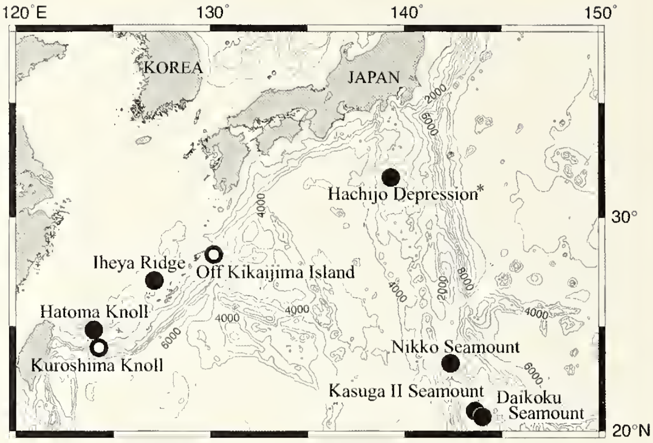


Figure 1. Sampling sites. Solid circles indicate hydrothermal vent. Open circles indicate methane seep. *No active vent/seep area has been discovered but the possibility of hydrothermal activity has been suggested.

Gills merobranchial, adanal, without interspace, 6-8 on each side.

Distribution and type of habitat

Iheya Ridge and off Kikaijima Island, Nansei Islands, 1395-1442 m; hydrothermal vent and methane seep.

Remarks

This species was described based on a single specimen with heavily eroded valves missing a large part of the tegmental sculpture. The remaining small portion of sculpturing and other features, especially the characteristic radula with elongate "toothpick"-like inner small (third) lateral, allows the additional specimens to be identified as this species.

The holotype was collected from undersurface of a rock, whereas all additional specimens were attached on the shells of *Bathymodiolus platifrons*.

Family Protochitonidae Ashby, 1925
Genus *Deshayesiella* Carpenter in Dall, 1879

Type species

Deshayesiella (Leptochiton) curvatus (Carpenter MS) Dall, 1879 (nom. nud., = *Lepidopleurus (Deshayesiella) curvatus* Carpenter in Pilsbry, 1892), by subsequent designation (Pilsbry, 1892).

Deshayesiella sirenkoi sp. nov.
(Figs. 3, 4, 5A-D)

Type material examined

Holotype: NSMT-Mo 73841 (ex. JAMSTEC sample No.: RK8-B-1, 006983), body length 36.4 mm. Type locality: hy-

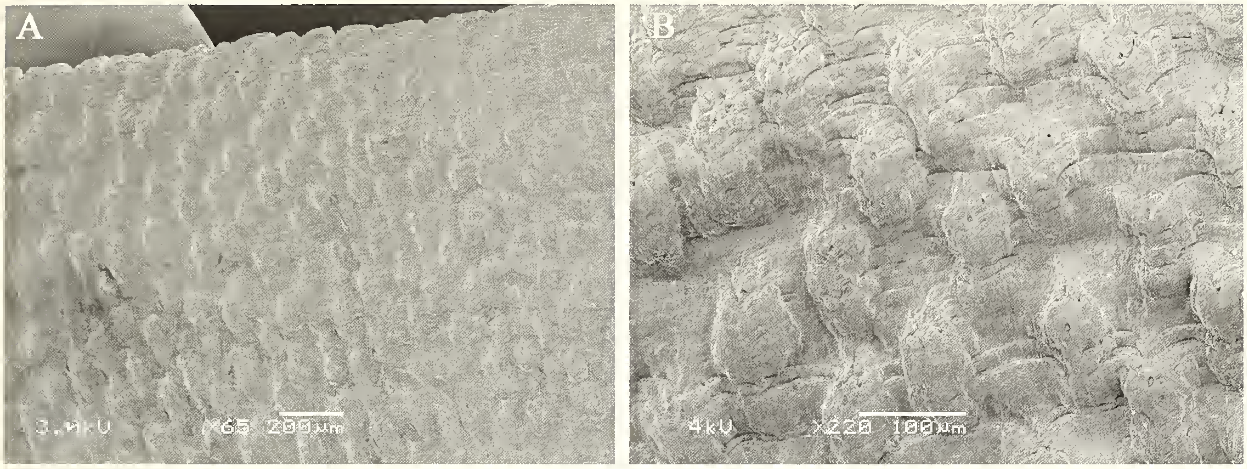


Figure 2. *Leptochiton tenuidontus* Saito and Okutani, 1991 (NSMT-Mo 73940) valve VI. A, sculpture on central area, scale bar = 200 µm; B, close-up of granules on central area, scale bar = 100 µm.

drothermal vent site on the Kasuga II Seamount in the northern Mariana Islands area, 21°36.1′N, 143°38.5′E, 400 m, 2K, Dive #986, 23 November 1997; paratypes: NSMT-Mo 73842, 1 specimen, body length ca. 45 mm, hydrothermal vent site on the Nikko Seamount in the northern Mariana Islands area, 23°04.7′N, 142°19.9′E, 460 m, 6K, Dive #144, 19 September 1992; NSMT-Mo 73843 (ex. JAMSTEC sample No.: FZ10, 061632-061635), 4 specimens, body length ca. 27-31 mm, hydrothermal vent site on the Daikoku Seamount in the northern Mariana Islands area, 21°19.53′N, 144°11.51′E, 428 m, on rock, HPD, Dive #498, 1 November 2005.

Diagnosis

Valves thick, low, slightly carinated. Median valves wide, angulated at antero-lateral corners, weakly protruded at anterior margin of jugal area. Tail valve with slightly raised mucro located anterior to the center, and concave posterior slope. Pleural areas sculptured with longitudinal, weakly curving riblets. Girdle with long needles.

Description

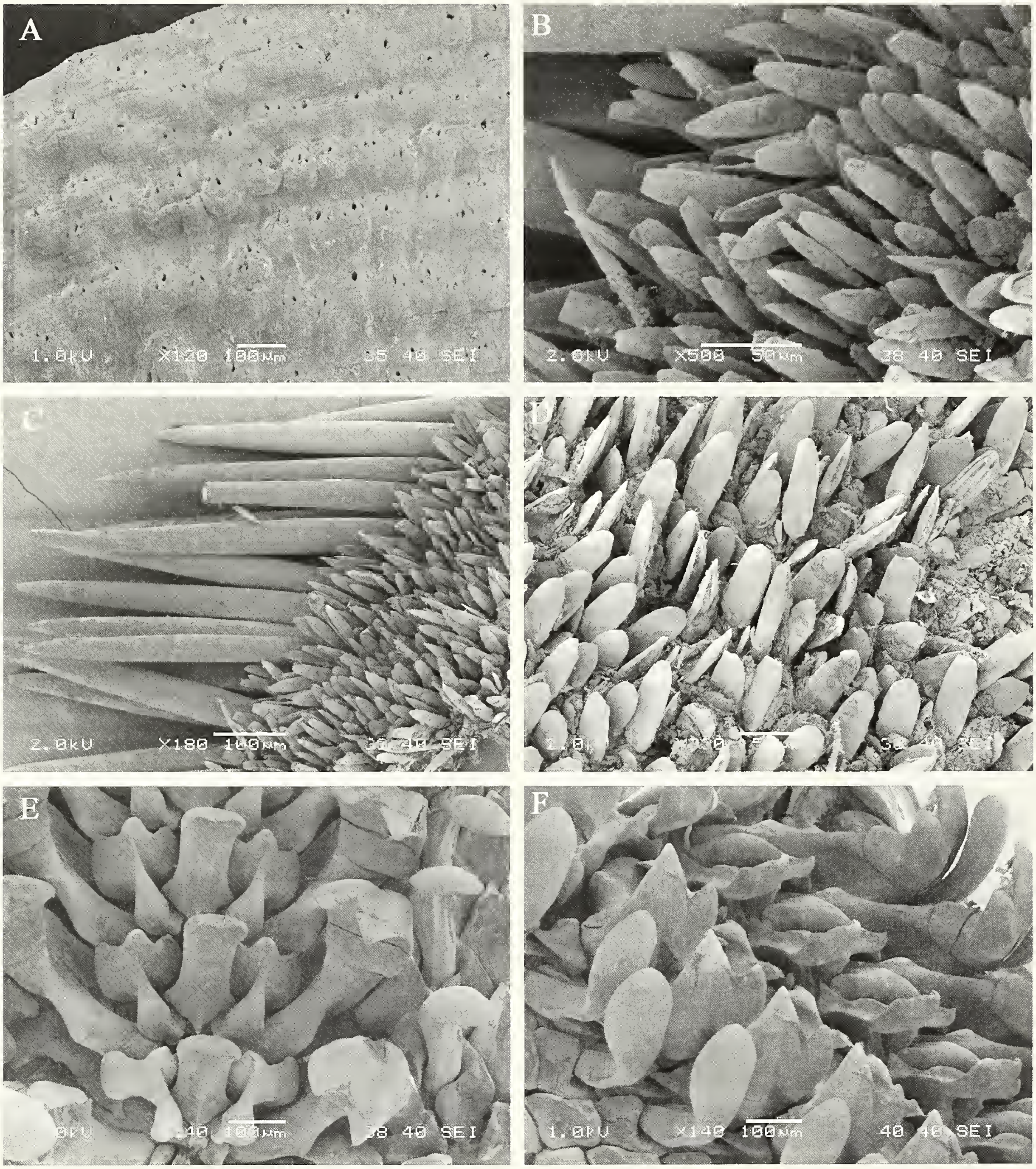
Body (Fig. 5A) oval, 36.4 mm in length. Valves (Fig. 5B) thick, low, slightly carinated. Girdle fleshy, deeply encroaching at sutures.

Head valve semicircular, rounded at postero-lateral corners. Median valves wide, widest at valves IV-VI, slightly carinated, beaked, weakly projected at anterior margin of jugal area. Tail valve more than semicircular, wider than head valve; mucro slightly raised, located anterior to the center; posterior slope concave. Tegmentum granulo-costate. Head valve, lateral areas of median valves, and posterior area of tail valve sculptured with densely packed granules which are often fused radially, forming larger elongate granules, marked with concentric growth lines; pleural areas of median and tail valves sculptured with strong, longitudinal, slightly curving riblets; jugal area densely sculptured with finer granules. Aesthete pores (Fig. 3A) located on anterior portion of each granule. Each group of pores consisting of one macraesthete pore, 10-20 µm in diameter, and one or two micraesthete pores, 5-8 µm in diameter at both sides of macraesthete pore. Articulamentum of head valve thickened, weakly projecting around the anterior margin of transverse muscle scars. Median valves and tail valve with widely V-shaped callus. Eaves wide, nearly smooth, scattered with minute aesthete pores. Tegmentum broadly folded under on posterior margin. Sutural laminae (Fig. 5B) strongly projected forward, triangular, widely separated from each other.

Girdle fleshy, thick, brownish. Perinotum (Figs. 3B, 5D) densely covered with elongate, obtusely pointed, flattened, distally ribbed spicules (Fig. 4A), 130 µm × 25 µm, intermingled with long, straight, smooth needles (Fig. 4B), up to 680 µm × 55 µm. Girdle margin fringed with long needles similar to those on perinotum (Fig. 3C). Spicules on hyponotum (Figs. 3D, 4C-E) flat with one to three strong riblets, 90 µm × 30 µm.

Gills merobranchial, adanal, without interspace, 16 on left, 18 on right.

Radula (Fig. 3E-F) long, 15.5 mm in length with 56 transverse rows of mineralized teeth. Central tooth oblong with narrow cusp at top, weakly expanded laterally, keeled near base. Centro-lateral (first lateral) teeth with well developed plate surrounding base of major lateral (second lateral) teeth, obtusely pointed at antero-dorsal corner. Major lateral teeth with bicuspid head, of which the larger outer cusp is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B
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C
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D
E
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F
1.0kV ×140 100µm 40 40 SEI

Figure 3. *Deshayesiella sirenkoi* sp. nov., holotype (NSMT-Mo 73841). A, valve II, granules on pleural area near anterior margin of jugal area, scale bar = 100 µm; B, spicules of perinotum near girdle margin, scale bar = 50 µm; C, marginal spicules, scale bar = 100 µm; D, spicules of hyponotum, scale bar = 50 µm; E, radula, central part, postero-dorsal view, scale bar = 100 µm; F, radula, central part, oblique antero-dorsal view, scale bar = 100 µm.

pointed and the smaller, inner cusp is rounded. Major uncinus (fifth lateral) teeth rounded at top with blade of moderate width. Bolster (radular vesicle and cartilage) length 5.2 mm.

Distribution and type of habitat

Known from the seamounts on Kasuga II, Nikko, and Daikoku in the northern Mariana Islands area, 400-460 m; hydrothermal vent.

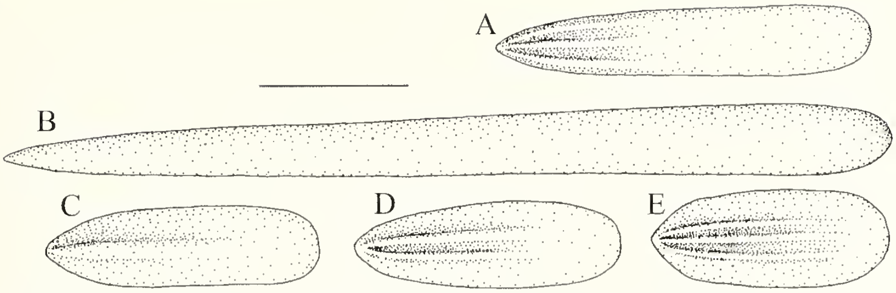

Figure 4. *Deshayesiella sirenkoi* sp. nov., holotype (NSMT-Mo 73841). A, spicule of perinotum; B, needle on perinotum; C-E, spicules of hyponotum; scale bar = 50 μm.

Etymology

This species is named in honor of Dr. Boris Sirenko, who has recently given a new diagnosis for the genus *Deshayesiella*.

Remarks

The features of the present species match the characteristics of *Deshayesiella* given by Sirenko (1997). These features include: valves solid, rather flat, evenly rounded; median valves divided into jugal, two pleural, and two lateral areas (unlike *Leptochiton*); tegmentum of head valve, lateral area of median valves and postmucronal area of tail valve sculptured with irregular granules, strongly marked with concentric lines of growth; girdle rather wide, dorsally covered with small spicules (100-150 μm) and randomly dispersed large spines (320-550 μm); radula with bicuspid major lateral teeth. Although there are some slight differences, such as the slightly carinated valves and somewhat longer large perinotal spines in the present species, we think they are insignificant for generic assignment. Sirenko (1997) recognized three known species in *Deshayesiella*: *D. curvata* (Carpenter in Pilsbry, 1892), *Oldroydia bidentata* Is. Taki, 1938, and *Hanleya sinica* Xu, 1990, as well as two undescribed species (sp. 1 and 2). The assignment of *H. sinica* may, however, need reconsideration because it has rather vaguely regionalized tegmentum with finer sculpture, and thus is more like members of *Leptochiton* in this respect. The present species is easily distinguishable from all known congeners and one of Sirenko's undescribed species, sp. 1, in having wider median valves, each side with more angular antero-lateral corner. The features of another undescribed species, sp. 2, have not yet been given in detail; however, the present new species is probably distinct from Sirenko's sp. 2 because the latter is distributed in a different geographic area: the East Pacific off southern California and in the Gulf of California, Mexico.

Deshayesiella sirenkoi is locally common around the hydrothermal vent site on the Daikoku Seamount (see Fig. 5C). *Deshayesiella sirenkoi*, as well as two known vent species, *Leptochiton tenuidontus* Saito and Okutani, 1990 and *Thermochiton undocostatus* Saito and Okutani, 1990 could be restricted to hydrothermal vent and/or methane seep habitats because each of these species was found only from those environments of more than two sites.

Order Chitonida Thiele, 1909
Suborder Chitonina Thiele, 1909
Family Ischnochitonidae Dall, 1889
Genus *Thermochiton* Saito and Okutani, 1990

Type species

Thermochiton undocostatus Saito and Okutani, 1990, by original designation.

Thermochiton undocostatus Saito and Okutani, 1990

Thermochiton undocostatus Saito and Okutani 1990: 171-174, figs. 13-23, pl. 2, figs. 1-4; Kaas and Van Belle 1994: 36-38, fig. 13; Kaas and Van Belle 1998: 192; Saito 2000: 11, pl. 6, fig. 12; Cosel 2006: 80.

Type material examined

Holotype: NSMT-Mo 69194, body length ca. 13 mm. Type locality: hydrothermal vent site on the Iheya Ridge, central Okinawa Trough, East China Sea, 27°32.70′N, 126°58.20′E, 1395 m, 2K, Dive #426, 21 July 1989.

Additional material examined

NSMT-Mo 73844 (ex. JAMSTEC sample No.: RK4-A-6, 038998-039004), 6 specimens, body length ca. 3-8 mm, Hydrothermal vent site on the Hatoma Knoll, 24°51.65′N, 123°50.29E, 1497 m, on small chimney rock, 2K Dive #1277, 29 May 2001; NSMT-Mo 73845, 2 specimens, body length ca. 7 mm, methane seep site on the Kuroshima Knoll off Yaeyama Islands area, 24°08.00′N, 124°11.50′E, 686-688 m, on the shells of *Bathymodiolus hirtus* Okutani, Fujikura, and Sasaki, 2004 or *Bathymodiolus securiformis* Okutani, Fujikura and Sasaki, 2004, and *Calyptogena kawamurai* (Kuroda, 1943), HPD, Dive #554, 21 May 2002.

Additional description

Gills nearly holobranchial (anterior-most gill located under the third valve), adanal, with interspace, 19 gills on each side (NSMT-Mo 73845).

Distribution and type of habitat

Off southern Nansei Islands, 686-1497 m; hydrothermal vent and methane seep.

Remarks

Characteristic features of the present specimens, such as undulating sculpture on the valves, granulo-costate dorsal

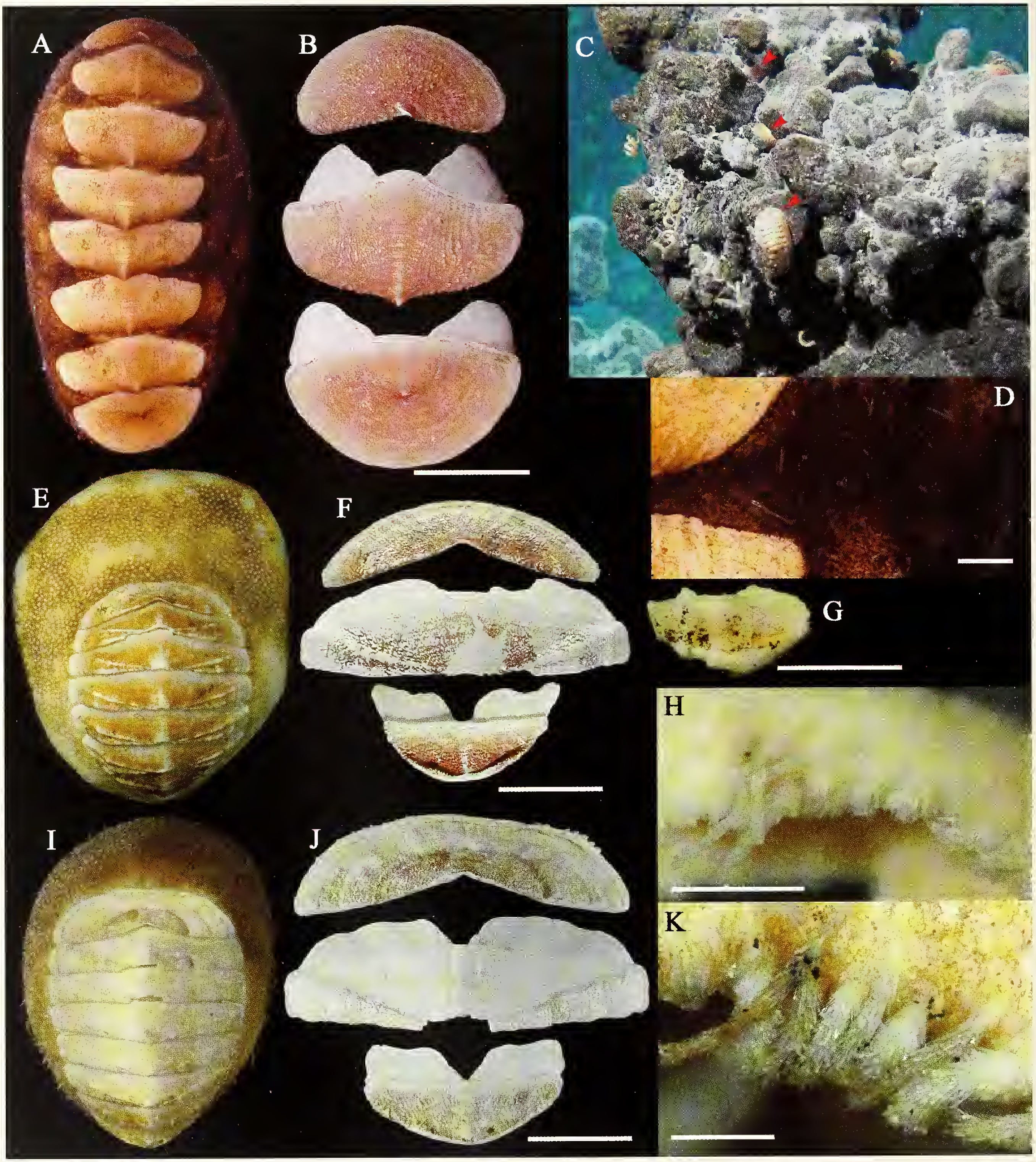

Figure 5. New species of chitons. A-D, *Deshayesiella sirenkoi*; E-H, *Placiphorella okutanii*; I-K, *Placiphorella isaotakii*. A, E, I, whole animal, dorsal view, holotypes; B, F, J, head, median (B: valve II; F, J: valve III), and tail valves, dorsal view, holotypes, scale bar = 5 mm; C, habitat, Daikoku Seamount, arrow heads indicate position of chitons; D, perinotum, showing long needles, holotype, scale bar = 1 mm; G, tail valve, paratype, scale bar = 1 mm; H, K, anterior margin of girdle, paratype and holotype, respectively, scale bar = 1 mm.

scales, and head of the major lateral tooth of radula with basal pointed projection agree well with those of the holotype.

Suborder Acanthochitonina Bergenhayn, 1930
Family Mopaliidae Dall, 1889
Genus *Placiphorella* Dall, 1879

Type species

Placiphorella velata (Carpenter MS) Dall, 1879, by original designation.

Placiphorella okutanii sp. nov.
(Figs. 5E-H, 6, 7)

Placiphorella stimpsoni: Wu and Okutani 1985: 126-128, figs. 9-18 (not of Gould 1859).

Type material examined

Holotype: NSMT-Mo 73777 (ex. JAMSTEC sample No.: RK4-B-5, 006454), body length 32 mm. Type locality: Hachijo Depression in the Izu-Ogasawara (Bonin) Islands area. 32°48.8′N, 139°27.0′E to 32°51.3′N, 139°31.6′E, 926-817 m, dredge attached to JAMSTEC Deep Tow Camera, cruise No.: DK88-3-IZU, 27 August 1988; paratype: NSMT-Mo 60008, body length ca. 30 mm, off Miyake Island, Izu-Ogasawara Islands area, 34°03.0′N, 140°02.2′E, 1210-1235 m, R/V *Soyo-Maru* St. B2, beam trawl, 5 July 1967.

Diagnosis

Valves chalky white, fragile, sculptured with densely packed, low, rather large, granules. Tail valve with narrow postmucronal areas separated by shallow sinus behind mucro. Sutural laminae wide, narrowly separated from each other. Perinotum densely implanted with low spiny tufts. Bristle implanted around girdle margin.

Description

Body (Fig. 5E) broadly oval, 32 mm in length, light buff in color.

Valves (Fig. 5F) wide, depressed, subcarinated, chalky white, fragile. Head valve crescent in outline, anterior slope concave. Median valves very wide, short, oblong in outline, weakly projected forward at jugal portion; lateral areas raised, grooved medially. Tail valve (Fig. 6A) small, inversed trapezoidal in outline, with narrow postmucronal area separated by shallow sinus at posterior end; mucro subterminal, slightly raised. Tegmentum (Fig. 5F) sculptured with densely packed, low, somewhat elongate granules on head valve, lateral areas of median valves, and postmucronal area of tail valve. Remaining tegmental areas with slightly lower, larger granules. Aesthete pores minute, 3-6 μm in diameter, distributed both on granules and the tegmental plain (Fig. 6B), which become denser toward the lateral areas (Fig. 6D). The difference between macraesthete pore and micraesthete pore hardly discernible. Articulamentum well developed, white, heavily calloused anteriorly in head valve, transversely in median valve, and posteriorly in tail valve; posterior margin of articulamentum widely covered with folded tegmentum in head and median valves, narrowly covered in tail valve. Sutural laminae well developed, narrowly separated from each other. Insertion teeth short, thick, rugose on anterior surface, with 12 slits in head valve (Fig. 6C), one on each side in median valves, none on tail valve. Slit rays represented by series of minute pores, clearly visible in apical half of head valve, median valves, inconspicuous in tail valve. Eaves narrow, with many minute pores.

Girdle (Fig. 5E) widely expanded anteriorly, becoming narrower toward posterior end. Perinotum (Fig. 6E) covered with minute spicules (Fig. 7A-B), mammilated at tip, ca. 150 μm × 30 μm, and densely implanted with low spiny tufts consist of 5-10 sharply pointed, weakly curved spicules (Fig. 7C-D), ca. 400 μm × 50 μm in width, surrounded by broken short spicules. Bristle, worn off in holotype, with sharply pointed spicules similar to spiny tufts. Hyponotum clothed with obtusely pointed, smooth spicules (Figs. 6F, 7G), 140-165 μm × 30 μm. Anterior hyponotum with numerous warts, which are provided with 20-30 pointed spicules (Fig. 7H-I), 150-170 μm × 25 μm. Pallial fold well developed with 9 precephalic tentacles, which are occasionally bifurcated. Spicules on pallial fold similar to obtusely pointed spicules on hyponotum, but smaller on precephalic tentacles, 110 μm × 15 μm (Fig. 7J), somewhat narrower on posterior end, 160 μm × 25 μm (Fig. 7K).

Gills holobranchial, abanal, with interspace, 15 on left side, 16 on right.

Radula (Fig. 6G-H) small, 6.5 mm in length, with 40 transverse rows of mineralized teeth. Central tooth oblong, with narrow cutting edge, slightly expanded laterally and bilobed at base. Centro-lateral (first lateral) teeth low, thickened at antero-dorsal corner. Major lateral (second lateral) teeth with proportionally small tridentate head. Major uncinus (fifth lateral) teeth with rather long blade of moderate width. Bolster (radular vesicle and cartilage) length 3.3 mm.

Paratype: Tail valve (Fig. 5G) with narrow posterior areas separated by shallow sinus at posterior end.

Bristle densely implanted along girdle margin (Fig. 5H). Thick bristles (Fig. 7E), ca. 300 μm in width, implanted on girdle margin and apparently thinner bristles (Fig. 7F), attaining ca. 1 mm × 100 μm, restricted on dorsal surface close to girdle margin, and occasionally on other area on perinotum.

Distribution and type of habitat

Only known from Hachijo Depression and off Miyake Island in Izu-Ogasawara Islands area, 817-1235 m; un-

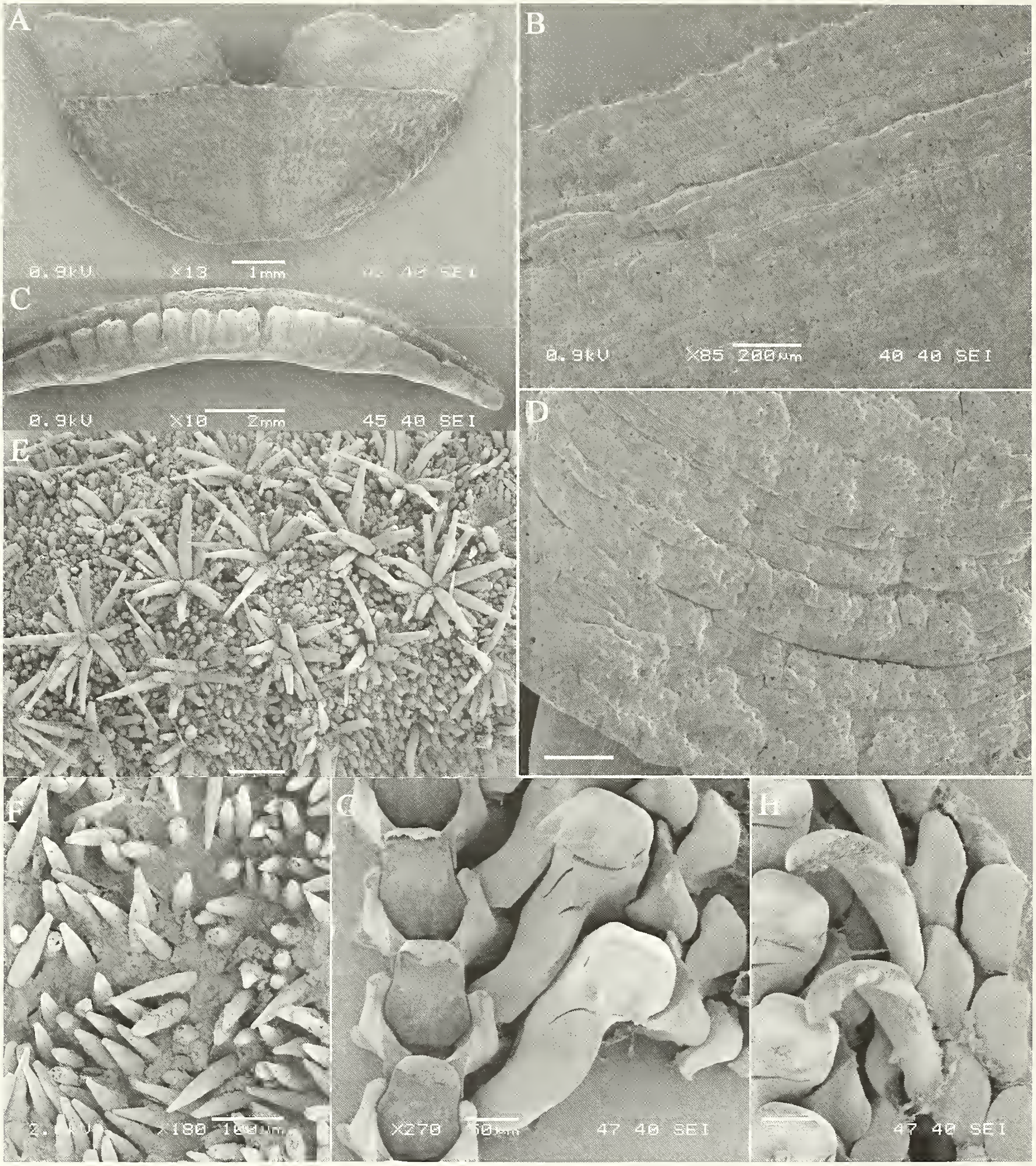
A
0.9kV X13 1mm
B
0.9kV X85 200µm 40 40 SEI
C
0.9kV X10 2mm 45 40 SEI
D
E
F
X180 100µm
G
X270 50µm 47 40 SEI
H
47 40 SEI

Figure 6. *Placiphorella okutanii* sp. nov., holotype (NSMT-Mo 73777). A, tail valve, scale bar = 1 mm; B, tegmentum of valve III, anterior margin of central area, scale bar = 200 µm; C, insertion teeth of head valve, scale bar = 2 mm; D, tegmentum of valve III, lateral area, scale bar = 200 µm; E, anterior perinotum, scale bar = 200 µm; F, spicules of hyponotum, scale bar = 100 µm; G, radula, central part (right half), postero-dorsal view, scale bar = 50 µm; H, radula, lateral part, postero-dorsal view, scale bar = 50 µm.

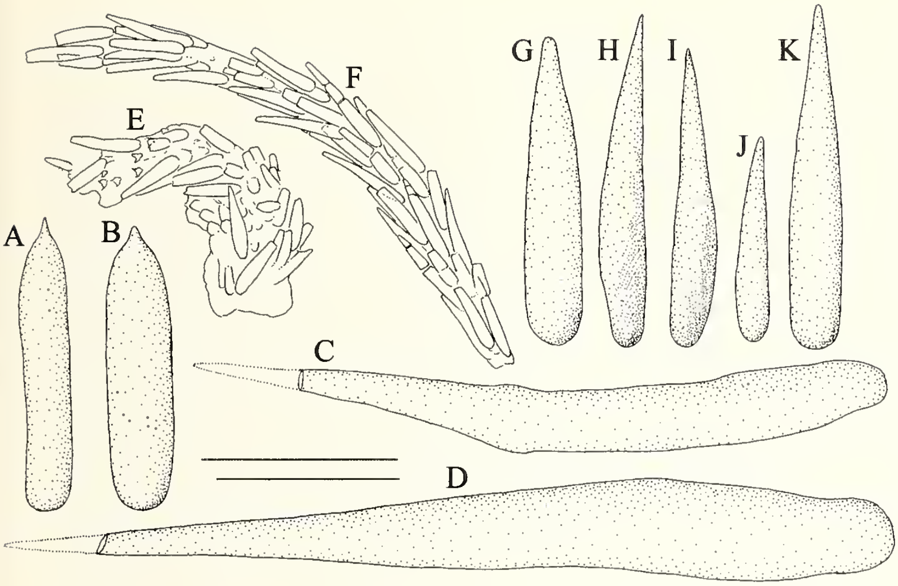

Figure 7. *Placiphorella okutanii* sp. nov., sclerites, A-D, G-K, holotype (NSMT-Mo 73777); E, F, paratype (NSMT-Mo 60008). A, B, spicules of perinotum; C, D, spicules of tuft on perinotum; E, thick bristle (spicules are lost in large part); F, thin bristle; G, spicules of hyponotum, H, I, spicules of tuft on hyponotum; J, spicule of precephalic tentacle; K, spicule of pallial fold near posterior end. Upper scale bar = 100 µm, for A-D, G-K; lower scale bar = 500 µm, for E and F.

known, possibility of hydrothermal activity is suggested in Hachijo Depression.

Etymology

This species is named in honor of Dr. Takashi Okutani, who has been actively working for deep-sea vent/seep molluscs, and collected this species for the first time.

Remarks

Kaas and Van Belle (1994) synonymized all known deep-sea *Placiphorella* species with *Placiphorella atlantica* (Verrill and Smith, 1882) and this decision was followed by Clark (1994). However, at least *Placiphorella pacifica* Berry, 1919 and *Placiphorella albitestae* Is. Taki, 1954 are distinctive, and can be separated by the valve shape and sculpture, girdle element shape and sclerite arrangement, and other features. Among those deep-sea *Placiphorella*, the present new species most closely resembles *Placiphorella "pacifica"* reported by Smith and Hanna (1952) from Pioneer Seamount, East Pacific, 500-650 m (CASIZ 064802) by having granular tegmentum. However, the granules of the former are irregular in shape and arrangement, especially on the lateral areas, and the spiny tufts of the perinotum are very prominent and dense. *Placiphorella "pacifica"* reported by Smith and Hanna (1952) can be an undescribed species because *P. pacifica* Berry, 1919 (Lectotype, SBMNH 34394 designated by Scott *et al.* 1990) has almost smooth surface on the tegmentum, and no other known species of *Placiphorella* has such an obviously granular tegmentum. From Japanese waters, another deep-sea species, *Placiphorella albitestae* was described from the Sagami Sea, northern Izu-Ogasawara Islands area. *Placiphorella albitestae* has much finer granules on finer, but sharply raised, growth lines on the tegmentum, much finer and scarce spinous tufts on the perinotum, and shallower bathymetrical range of distribution, from 80 to 200 m (Saito 2000).

Placiphorella isaotakii n. sp.
(Figs. 5I-K, 8, 9)

Type material examined

Holotype: NSMT-Mo 73778 (ex. JAMSTEC sample No.: RK4-A-3, 016481), body length ca. 34 mm. Type locality: methane seep sites on the Kuroshima Knoll off Yaeyama Islands area, 24°07.00′N, 124°11.00′E, 691-692 m, 2K, Dive #1100, 22 May 1999.

Diagnosis

Valves solid, sculptured with fine elongate granules. Tail valve wide triangular, with terminal mucro. Sutural laminae wide, narrowly separated each other. Insertion teeth low, hardly separated with slits in head valve. Perinotum densely implanted with low spinous tufts. Bristle implanted along girdle margin.

Description

Body (Fig. 5I) broadly oval, ca. 34 mm in length, light buff in color.

Valves (Fig. 5J) wide, depressed, subcarinated, solid. Head valve crescent in outline; anterior slope concave. Median valves very wide, short, oblong in outline, weakly projected forward at jugal portion; lateral areas raised, grooved medially. Tail valve (Fig. 8A) small, wide triangular in outline, with terminal mucro. Tegmentum (Fig. 5J) sculptured with densely packed, elongate granules on head valve and lateral areas of median valves. Remaining tegmental areas with weak, elongated granules, which are occasionally merged into longitudinal threads. Aesthete pores minute, 3-5 µm in diameter, arranged roughly in concentric patterns

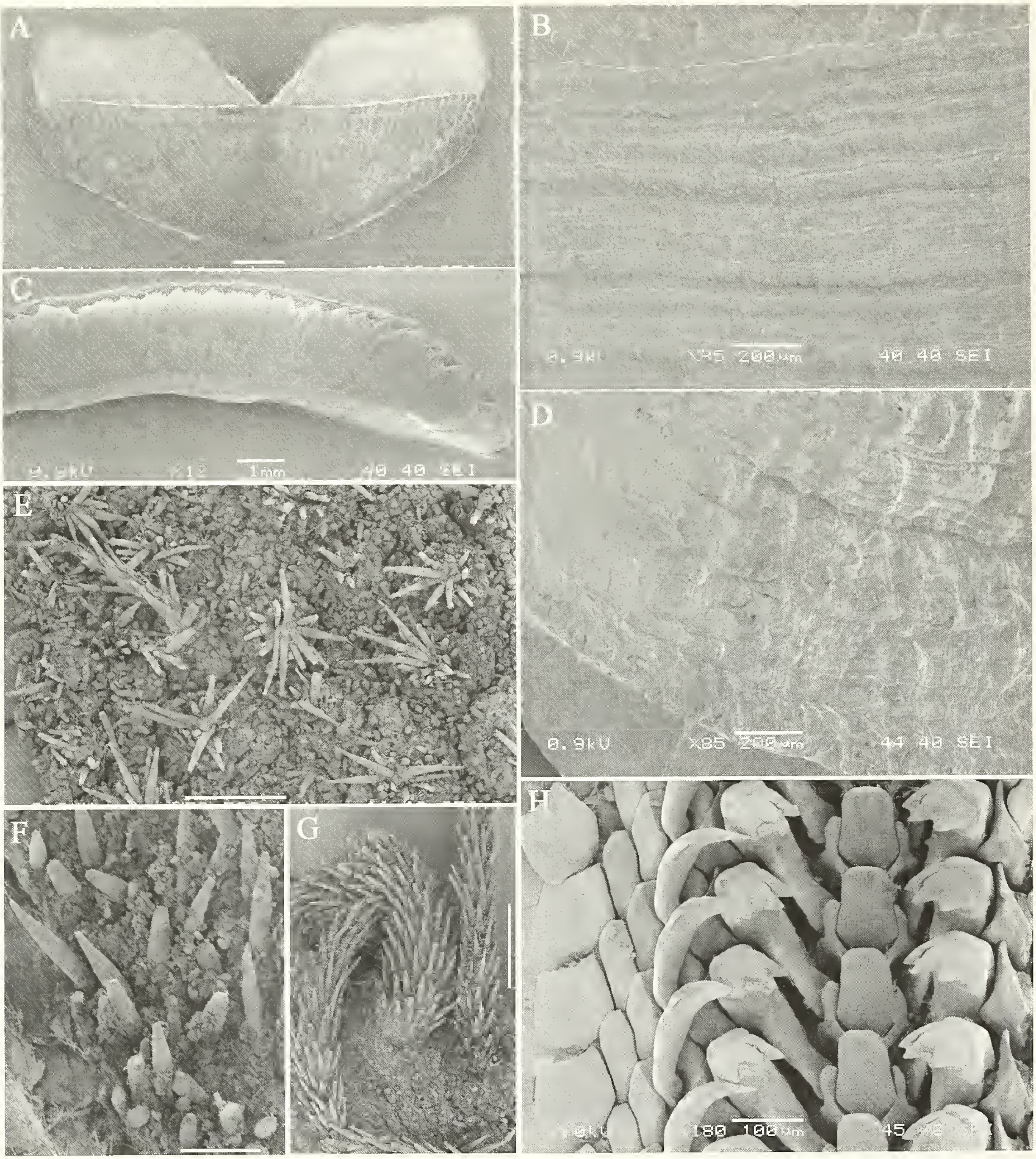

Figure 8. *Placiphorella isaotakii* sp. nov., holotype (NSMT-Mo 73778). A, tail valve, scale bar = 1 mm; B, tegmentum of valve III, anterior margin of central area, scale bar = 200 µm; C, insertion teeth of head valve, scale bar = 1 mm; D, tegmentum of valve III, lateral area, scale bar = 200 µm; E, perinotum, scale bar = 500 µm; F, spicules of hyponotum, scale bar = 100 µm; G, bristles near anterior margin, scale bar = 500 µm; H, radula, postero-dorsal view, scale bar = 100 µm.

in pleural areas (Fig. 8B), which are denser and less regularly arranged on lateral areas (Fig. 8D). Difference between macraesthete pore and micraesthete pore hardly discernible. Articulamentum well developed, white, heavily calloused anteriorly in head valve, transversely in median valve, and posteriorly in tail valve; posterior margin of articulamentum widely covered with folded tegmentum in all valves. Sutural laminae well developed, narrowly separated from each other.

Insertion teeth low, thick, rugose on outside. Slits inconspicuous on head valve (Fig. 8C), one on each side in median valves, none in tail valve. Slit rays inconspicuous, represented by minute pores. Eaves narrow, with minute pores.

Girdle (Fig. 5I) widely expanded anteriorly, becoming narrower toward posterior end. Perinotum (Fig. 8E) covered with minute, thick spicules (Fig. 9A-B), obtuse or weakly mammilated at tip, 150 µm × 40 µm, and densely implanted with low spinous tufts consist of 5-10 sharply pointed spicules (Fig. 9C-D), 440 µm × 50 µm surrounded by broken short spicules. Thick bristle (Figs. 8G, 9E), up to 2.5 mm × 400 µm, implanted along the girdle margin, while thinner bristle (Fig. 9F) restricted on dorsal surface close to girdle margin (Fig. 5K) and occasionally on other area of perinotum. Spicules on bristle similar to those of tufts, but less curved and slightly shorter, 380 µm × 50 µm. Hyponotum clothed with obtusely pointed, smooth spicules (Figs. 8F, 9G-H), attaining 180 µm × 30 µm. Anterior hyponotum with numerous warts which are provided with 20-40 pointed spicules (Fig. 9I), 150-170 µm × 25 µm. Pallial fold well developed with nine precephalic tentacles, which are occasionally bifurcated. Spicules on pallial fold similar to obtusely pointed spicules on hyponotum, but smaller on precephalic tentacles, 120 µm × 15 µm (Fig. 9J), somewhat narrower on posterior end, 165 µm × 25 µm (Fig. 9K).

Gills holobranchial, abanal, with interspace, 21 on left side, 22 on right.

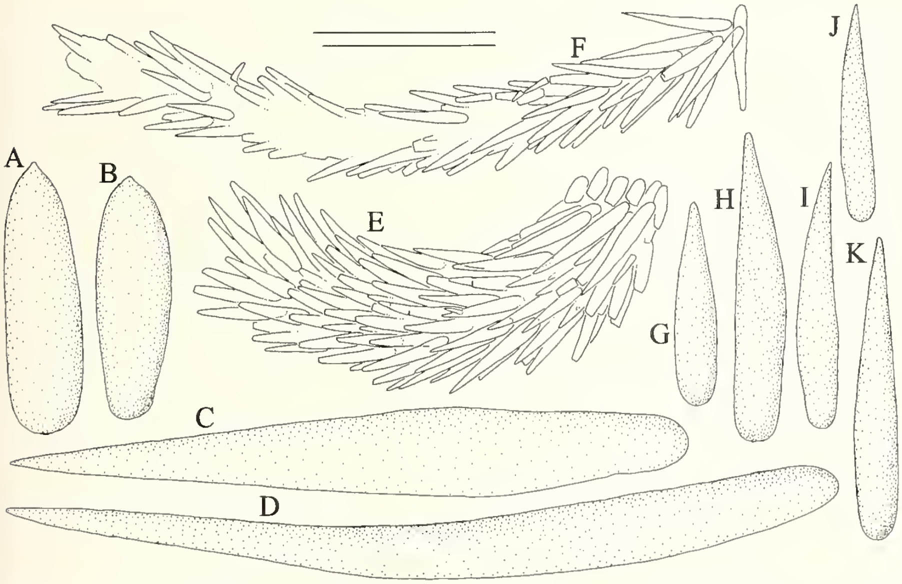

Figure 9. *Placiphorella isaotakii* sp. nov., sclerites, holotype (NSMT-Mo 73778). A, B, spicules of perinotum; C, D, spicules of tuft on perinotum; E, thick bristle; F, thin bristle (some parts are not traced due to foreign deposit); G, H, spicules of hyponotum; I, spicules of tuft on hyponotum; J, spicule of precephalic tentacle; K, spicule of pallial fold near posterior end. Upper scale bar = 100 µm, for A-D, G-K; lower scale bar = 500 µm, for E, F.

Radula (Fig. 8H) small, 7.5 mm in length, with 41 transverse rows of mineralized teeth. Central tooth oblong, with narrow cutting edge, slightly expanded laterally and bilobed at base. Centro-lateral (first lateral) teeth low, thickened at antero-dorsal corner. Major lateral (second lateral) teeth with small tridentate head. Major uncinus (fifth lateral) teeth with rather long blade of moderate width. Bolster (radular vesicle and cartilage) length 3.4 mm.

Distribution and type of habitat

Known only from the type locality; methane seep.

Etymology

This species is named in honor of the late Dr. Isao Taki, who described the first deep-sea *Placiphorella*, *P. albitestae* from Japanese waters.

Remarks

This species also resembles *Placiphorella* "*pacifica*" reported by Smith and Hanna (1952) and the preceding new species, *Placiphorella* by having a granular tegmentum; however, the present species differs by having a terminal mucro on a wider tail valve, and thread-like sculpture of the central area.

The two new *Placiphorella* species described here might be transient species, rather than vent/seep specialists because *Placiphorella* species have been shown to be carnivorous, using their anterior expanded girdle to trap prey (McLean 1962, Saito and Okutani 1992). They may be able to live in non-chemosynthetic environment if enough prey were available.

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INDEX TO VOLUME 25

AUTHOR INDEX

Bidwell, J. R. 25: 1
Binks, R. M. 25: 35
Boeckman, C. J. 25: 1
Brooker, L. R. 25: 35
Buckland-Nicks, J. 25: 97
Cacabelos, E. 25: 9
Clark, R. N. 25: 71, 77
Clode, P. L. 25: 35
DuFoe, J. 25: 25
Eernisse, D. J. 25: 21, 51, 87
Fernandez, C. Z. 25: 51
Fujikura, K. 25: 113
Johnson, C. C. 25: 87
Macey, D. J. 25: 35
Pojeta, J., Jr. 25: 25
Puchalski, S. P. 25: 87
Quintas, P. 25: 9
Runnegar, B. 25: 51
Saito, H. 25: 113
Shaw, J. A. 25: 35
Sigwart, J. D. 25: 43
Stockdale, E. J. 25: 35
Troncoso, J. S. 25: 9
Tsuchida, S. 25: 113
Vendrasco, M. J. 25: 51
Webb, R. I. 25: 35

PRIMARY MOLLUSCAN TAXA INDEX

[first occurrence in each paper recorded, new taxa in bold]

aberti, Cyprogenia 25: 1
abyssicola, Ischnochiton 25: 81
abyssicola, Tripoplax 25: 77
Acaenoplax 25: 33
Acanthochitona 25: 110
Acanthochitonidae 25: 98
Acanthochitonina 25: 51, 72, 97, 119
Acanthopleura 25: 52, 98
Acanthopleuridae 25: 98
acuta, Mopalia 25: 53
alba, Abra 25: 9
albitestae, Placiphorella 25: 121
Albrechtia 25: 78
albus, Ishnochiton 25: 98
albus, Stenosemus 25: 97
algesirensis, Leptochiton 25: 45
allyni, Tripoplax 25: 86
alveolus, Leptochiton 25: 113
Amicula 25: 52
antiqua, Diadeloplax 25: 88
antiquus, Chitonellus 25: 88
apiculata, Chaetopleura 25: 97
Aplacophora 25: 33, 108
argenteum, Chaetoderma 25: 109
asellus, Leptochiton 25: 43, 99, 114
asiatica, Hanleyella 25: 43
assimilis, Leptochiton 25: 43, 98
atlantica, Placiphorella 25: 121
attuensis, Tripoplax 25: 86
aurata, Plaxiphora 25: 51
australis, Chiton 25: 77
australis, Epimenia 25: 109
australis, Ischnochiton 25: 78
badius, Leptochiton 25: 45
balenophila, Lepidozona 25: 113
baxteri, Tripoplax 25: 86
belknapi, Leptochiton 25: 45
beringiana, Tripoplax 25: 86
bidentata, Mysella 25: 9
bidentata, Oldroydia 25: 117
cajetanus, Lepidopleurus 25: 45, 53
californica, Nuttallina 25: 53, 98
Callistoplacidae 25: 98
Callochiton 25: 75, 97
Callochitonidae 25: 97
calypso, Tripoplax 25: 77
cancellatus, Leptochiton 25: 45
cardium, Lampsilis 25: 2
castaneus, Callochiton 25: 99
Chaetopleura 25: 110
Chaetopleurinae 25: 52, 110
Chelodes 25: 88
Chelodida 25: 31
chilensis, Tonicia 25: 51
chinensis, Calyptraea 25: 12
Chitonida 25: 43, 97, 117
Chitonidae 25: 51
Chitonina 25: 52, 97, 117
Choriplacidae 25: 98
ciliata, Chiton 25: 74
ciliata, Mopalia 25: 23, 51, 71
cinerea, Cyanoplax 25: 110
cinerea, Lepidochitona 25: 51, 110
cinereus, Chiton 25: 114
cinereus, Trachydermon 25: 51
coccineum, Pleuribema 25: 2
complanata, Lasmigona 25: 2
conspicua, Stenoplax 25: 97
cooperi, Lepidozona 25: 53
cordiformis, Chiton 25: 88
cowani, Tripoplax 25: 77
Cryptochiton 25: 52
Cryptoplacidae 25: 98
curvata, Deshayesiella 25: 43, 98, 117
curvatus, Deshayesiella 25: 114
curvatus, Lepidopleurus 25: 114
Cyanoplax 25: 54, 97
cylindrica, Quadrula 25: 1
davolii, Lepidopleurus 25: 88
decussatus, Tapes 25: 9
Dendrochiton 25: 51
dentatus, Callochiton 25: 98
dentiens, Cyanoplax 25: 98
dentiens, Lepidochiton 25: 98
Deshayesiella 25: 108, 113
distortus, Chitonellus 25: 88
donaciformis, Truncilla 25: 2
dufoei, Echinochiton 25: 21, 25
echinatus, Acanthopleura 25: 110
Echinochiton 25: 31

Echinochitonidae 25: 31
edule, Cerastoderma 25: 9
edulis, Ostrea 25: 12
Eopteria 25: 25
eugenei, Parachiton 25: 45
exiguum, Parvicardium 25: 9
fenestrata, Chrysallida 25: 12
fernaldi, Cyanoplax 25: 98
fernaldi, Lepidochitona 25: 98
Ferreiraellidae 25: 98
flava, Fusconaia 25: 2
flectens, Dendrochiton 25: 51
flexuosa, Thyasira 25: 9
foresti, Leptochiton 25: 45
fragilis, Leptodea 25: 2
gibba, Corbula 25: 12
Glyptochiton 25: 88
golischi, Lepidozona 25: 77
Gotlandochiton 25: 88
granulata, Acanthopleura 25: 98
grayii, Mopalia 25: 72
Gurjanovillia 25: 78
hakodadensis, Ischnochiton 25: 109
hancockianus, Chitonellus 25: 88
Hanleya 25: 108
hanleyi, Hanleya 25: 98
hartwegii, Cyanoplax 25: 53
hartwegii, Lepidochitona 25: 53
hayae, Acaenoplax 25: 33
Helminthochiton 25: 88
Hemiarthridae 25: 98
hindsii, Chiton 25: 72
hindsii, Mopalia 25: 71
hirtosa, Acanthopleura 25: 35
hirtus, Bathymodiolus 25: 117
hyadesi, Nuttallochiton 25: 53
hylkiae, Parachiton 25: 45
ima, Tripoplax 25: 86
incisus, Schizochiton 25: 52
insignis, Tonicella 25: 51
isaotakii, Placiphorella 25: 113
Ischnochiton 25: 54, 97
Ischnochitonidae 25: 52, 77, 98, 117
Ischnoradsia 25: 77
Katharina 25: 52
kawamurai, Calyptogena 25: 117
kennerleyi, Mopalia 25: 23, 71
kurnilatus, Leptochiton 25: 45
labiosa, Rissoa 25: 9
lacteus, Loripes 25: 9
Lepidochitona 25: 52, 78, 97
Lepidochitonidae 25: 52, 98
Lepidopleurida 25: 23, 43, 97, 114
Lepidopleuridae 25: 43, 98
Lepidopleurina 25: 23, 43, 114
Lepidopleurus 25: 43, 54, 89
Lepidozona 25: 54, 77, 97
Leptochiton 25: 43, 81, 113
Leptochitonidae 25: 43, 98, 114
lignosa, Mopalia 25: 51
lineata, Nierstraszella 25: 45
lineata, Tonicella 25: 51, 109
lirulatus, Dendrochiton 25: 51
lokii, Tonicella 25: 51
Loricidae 25: 98
Macoma 25: 9
marmorea, Tonicella 25: 51
Matthevia 25: 32
mertensii, Lepidozona 25: 53, 77
mesogonus, Leptochiton 25: 80
metanevra, Quadrula 25: 2
micropustulosus, Leptochiton 25: 45
mirandus, Nuttallochiton 25: 53
Mopalia 25: 52, 75, 104
Mopaliidae 25: 23, 51, 62, 72, 108
multiganosa, Lepidozona 25: 77
Multiplacophora 25: 32
Multiplacophora 25: 33
muscosa, Mopalia 25: 53, 72, 89, 104
neglectus, Onithochiton 25: 52
nervosa, Megalonaias 25: 2
Nierstraszella 25: 45
Nierstraszellidae 25: 98
nigrovirescens, Chiton 25: 98
nigrovirescens, Radsia 25: 98
nitida, Abra 25: 9
nitidosa, Nucula 25: 9
nitidum, Lepton 25: 12
nodulata, Quadrula 25: 2
norfolcensis, Leptochiton 25: 44
Nuttallochiton 25: 52
Odontogriphus 25: 21, 32
ohiensis, Potamilus 25: 4
okutanii, Placiphorella 25: 113
omalus, Odontogriphus 25: 32
pacifica, Placiphorella 25: 80, 121
Paleoloricata 25: 31
Parachiton 25: 45
pectinulata, Lepidozona 25: 53
philippina, Nierstraszella 25: 45
Placiphorella 25: 52, 113
Placiphorina 25: 52
plana, Scrobicularia 25: 14
platifrons, Bathymodiolus 25: 114
Plaxiphora 25: 51
plicata, Amblema 25: 2
politus, Parachiton 25: 45
polymorpha, Dreissena 25: 1
Polyplacophora 25: 21, 25, 35, 43, 51, 71, 77, 87, 97, 113
Polysacos 25: 33
Priscochiton 25: 88
Protochitonidae 25: 114
purpuratus, Potamilus 25: 2
pustulosa, Quadrula 25: 2
Quadrula 25: 1
quadrula, Quadrula 25: 4
radiata, Turboella 25: 9
reflexa, Obliquaria 25: 2
regularis, Ischnochiton 25: 86
retiporosa, Lepidozona 25: 77, 109
Rhyssoplax 25: 110
rissoi, Ischnochiton 25: 110
rugatus, Leptochiton 25: 43, 98
sarsi, Leptochiton 25: 45
scabricostata, Lepidozona 25: 80
Schizochiton 25: 97
Schizochitonidae 25: 51, 98
scrobiculata, Lepidozona 25: 77
securiformis, Bathymodiolus 25: 117
silesiacus, Pterochiton 25: 88
sinica, Hanleya 25: 117
sinudentata, Lepidozona 25: 80
sirenkoi, Deshayesiella 25: 113
spectabilis, Mopalia 25: 51, 75
spinifera, Myrtea 25: 12
stearnsii, Stenosemus 25: 77
stelleri, Cryptochiton 25: 55, 98
Stenoplax 25: 98
Stenosemus 25: 110, 113
stimpsoni, Placiphorella 25: 119
swanii, Mopalia 25: 51
tenuidontus, Leptochiton 25: 113
terebellum, Chrysallida 25: 10
teres, Lampsilis 25: 2
textilis, Ischnochiton 25: 53
Thermochiton 25: 117

thouarsiana, Chaetopleura 25: 72
Tonicella 25: 51, 104
Tonicellidae 25: 57, 97
Tonicia 25: 69
Toniciinae 25: 110
Trachydermon 25: 78
trifida, Tripoplax 25: 85
trifidus, Ischnochiton 25: 77
tripartitus, Pterochiton 25: 88
Tripoplax 25: 23, 77
truncatula, Retusa 25: 12
tuberculatus, Chiton 25: 52, 101
tulipa, Chiton 25: 98
tulipa, Rhyssoplax 25: 97
tunicata, Katharina 25: 51
ulvae, Hydrobia 25: 9
undocostatus, Thermochiton 25: 113
Unionidae 25: 1
variabilis, Matthevia 25: 32
variegatus, Ischnochiton 25: 53
velata, Placiphorella 25: 53, 119
verrucosa, Tritogonia 25: 2
vickersianum, Polysacos 25: 32
viridis, Acanthochitona 25: 98
vitrea, Hyala 25: 12
willetti, Lepidozona 25: 77
Wiwaxia 25: 21
wosnessenskii, Chiton 25: 71

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Mopalia kennerleyi Carpenter, 1864, a forgotten species and its southern analogue *Mopalia ciliata* (Sowerby, 1840). **ROGER N. CLARK** 71

Two new chitons of the genus *Tripoplax* Berry, 1919 from the Monterey Sea Canyon. **ROGER N. CLARK** 77

The effect of sampling bias on the fossil record of chitons (Mollusca, Polyplacophora). **STEPHANEY S. PUCHALSKI, DOUGLAS J. EERNISSE, and CLAUDIA C. JOHNSON** 87

Fertilization biology and the evolution of chitons. **JOHN BUCKLAND-NICKS** 97

Chitons (Mollusca: Polyplacophora) associated with hydrothermal vents and methane seeps around Japan, with descriptions of three new species. **HIROSHI SAITO, KATSUNORI FUJIKURA, and SHINJI TSUCHIDA** 113

Index to Vol. 25 125

Membership Form for 2008 128

Information for Contributors 130

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AMERICAN MALACOLOGICAL BULLETIN

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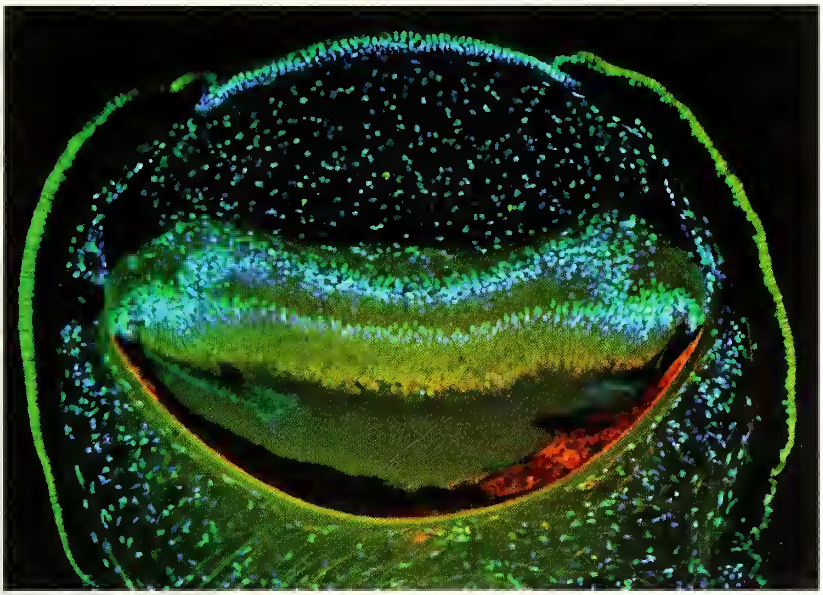
VOLUME 26 DECEMBER 29, 2008 NUMBER 1/2



Introduction to the symposium "Molluscan models: Advancing our understanding of the eye". **JEANNE M. SERB** 1

Toward developing models to study the disease, ecology, and evolution of the eye in Mollusca. **JEANNE M. SERB** 3

Rho signaling mediates cytoskeletal re-arrangements in octopus photoreceptors. **SHAUNTÉ M. GRAY, SHANNON KELLY, and LAURA J. ROBLES** 19

Comparative morphology of the concave mirror eyes of scallops (Pectinoidea). **DANIEL I. SPEISER and SÖNKE JOHNSEN** 27

The evolution of eyes in the Bivalvia: New insights. **BRIAN MORTON** 35

Understanding the cephalic eyes of pulmonate gastropods: A review. **MARINA V. ZIEGER and VICTOR BENNO MEYER-ROCHOW** 47

The genus *Buccinanops*: A model for eye loss in caenogastropods. **ANDRÉS AVERBUJ and PABLO E. PENCHASZADEH** 67

Evolution of mollusc lens crystallins: Glutathione S-transferase/S-crystallins and aldehyde dehydrogenase/Ω-crystallins. **JORAM PIATIGORSKY** 73

continued on back cover

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AMERICAN MALACOLOGICAL BULLETIN

CONTENTS

VOLUME 26 | NUMBER 1/2

Introduction to the symposium "Molluscan models: Advancing our understanding of the eye".
JEANNE M. SERB 1

Toward developing models to study the disease, ecology, and evolution of the eye in Mollusca.
JEANNE M. SERB 3

Rho signaling mediates cytoskeletal re-arrangements in octopus photoreceptors.
SHAUNTÉ M. GRAY, SHANNON KELLY, and LAURA J. ROBLES 19

Comparative morphology of the concave mirror eyes of scallops (Pectinoidea).
DANIEL I. SPEISER and SÖNKE JOHNSEN 27

The evolution of eyes in the Bivalvia: New insights. **BRIAN MORTON** 35

Understanding the cephalic eyes of pulmonate gastropods: A review.
MARINA V. ZIEGER and VICTOR BENNO MEYER-ROCHOW 47

The genus *Buccinanops*: A model for eye loss in caenogastropods.
ANDRÉS AVERBUJ and PABLO E. PENCHASZADEH 67

Evolution of mollusc lens crystallins: Glutathione S-transferase/S-crystallins and aldehyde dehydrogenase/Ω-crystallins. **JORAM PIATIGORSKY** 73

Photoreception and the polyphyletic evolution of photoreceptors (with special reference to Mollusca). **LUITFRIED VON SALVINI-PLAWEN** 83

Primary inhibition by light: A unique property of bivalve photoreceptors. **LON A. WILKENS** 101

When a snail dies in the forest, how long will the shell persist? Effect of dissolution and micro-bioerosion. **TIMOTHY A. PEARCE** 111

Bivalve molluscs from the continental shelf of Jalisco and Colima, Mexican Central Pacific.
EDUARDO RÍOS-JARA, ERNESTO LÓPEZ-URIARTE, and CRISTIAN M. GALVÁN-VILLA 119

A mature female of *Bathothauma* Chun, 1906 (Cephalopoda: Cranchiidae) from Hawaii.
JANET R. VOIGHT 133

Conservation of the freshwater gastropods of Indiana: Historic and current distributions.
MARK PYRON, JAYSON BEUGLY, ERIKA MARTIN, and MATTHEW SPIELMAN 137

The feeding behavior and diet of an endemic West Virginia land snail, *Triodopsis platysayoides*.
DANIEL C. DOURSON 153

Structural community changes in freshwater mussel populations of Little Mahoning Creek, Pennsylvania. **ERIC J. CHAPMAN and TAMARA A. SMITH** 161

Diversity and distribution of freshwater gastropods in the Bayou Bartholomew drainage, Arkansas, U.S.A. **RUSSELL L. MINTON, JOHN D. WHITE, DAVID M. HAYES, M. SEAN CHENOWETH, and ANNA M. HILL** 171

Research Note: *Conus lightbourni* holotype returned to the Delaware Museum of Natural History. **ELIZABETH K. SHEA and WILLIAM J. FENZAN** 179

Index to Vol. 26 181

Membership Form for 2009 185

Information for Contributors 187

Meeting Announcement 189

Saito *et al.* (2008) AMB **25:** 113-124.

Dourson (2008) AMB **26:** 153-159.

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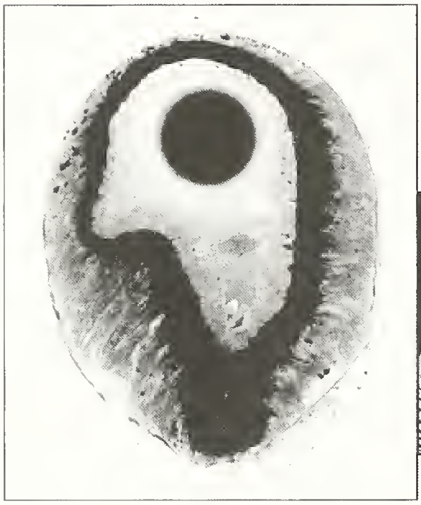
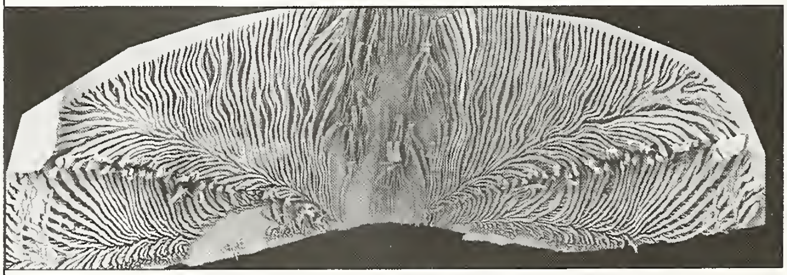
Zieger and Meyer-Rochow
(2008) AMB **26:** 47-66.





Vendrasco *et al.* (2008) AMB **25:** 51-69.

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Introduction to the symposium "Molluscan models: Advancing our understanding of the eye"*

Jeanne M. Serb

Department of Ecology, Evolution and Organismal Biology, 253 Bessey Hall, Iowa State University,
Ames, Iowa 50011, U.S.A., serb@iastate.edu

Since the time of Darwin, the eye has been a subject of evolutionary and comparative biologists alike who were intrigued by the structural complexity and morphological diversity of eyes in nature. Much of what we know about the eye—development, structure, physiology, and function—has been determined from only a handful of model organisms, specifically the mouse and the fly. One major phylum in particular, the Mollusca, has been underutilized in investigating the evolution and development of the eye. This is surprising as molluscs display a myriad of eye types, such as simple pit eyes without any apparatus to focus images, compound eyes that superficially resemble the eyes of flies, camera-type eyes that are similar to vertebrate eyes, and eyes with mirrors, just to name a few. As a result, molluscan eyes comprise more morphological diversity than seen even in the largest animal phylum, the Arthropoda.

With all of this incredible diversity, how do we as researchers determine which mollusc species should be developed as models to study the eye? Serb provides background for eye research using traditional model organisms and how using molluscan species would be advantageous to understanding the eye. She describes the research potential of molluscan species as model organisms and identifies criteria that might be used to develop a molluscan model and the questions molluscan models might address.

One application of molluscan models is to study the cellular biology of human eye disease. As many degenerative eye diseases, such as macular degeneration, have been linked to the mis-organization of the cytoskeleton within retinal cells, understanding the control of cytoskeleton organization and its influence on photoreceptor cell changes may lead to prevention and possible cures for some eye diseases. Gray, Kelly, and Robles utilize *Octopus bimaculoides* Pickford and McConnaughey, 1949 as a model organism to study the molecular controls of cytoskeleton organization in the retina. Their work identifies a cell signaling pathway (Rho GTPase) that mediates cytoskeleton rearrangements. Errors in this pathway may prove to be one of the factors that disrupts cytoskeleton formation, leading to retinal degeneration.

After developing one or several molluscan models of the eye, how does one set about understanding this great diversity of eyes and place it in an evolutionary context? One way is to use a comparative approach to identify conserved and variable components of eye morphology, such as lens composition, photoreceptor number and organization, and overall eye shape. These morphological features can provide evidence for functional differences and visual capabilities among species. Several authors in the symposium take this approach. Speiser and Johnsen examine eye morphology in four species of scallop and a closely related spondylid (*Spondylus americanus* [Hermann, 1781]). They show that scallop eye structure varies among species, and these structural differences affect optical resolution and sensitivity. Further, they provide evidence that actively swimming species (*e.g.*, *Amusium balloti* [Bernardi 1861]) have better optical resolution than non-swimming species. Speiser and Johnsen provide several new and exciting hypotheses on how the scallop eye performs and how visual requirements may differ between mobile and immobile species. Morton takes a broader perspective and reviews the diversity of non-cephalic eye types in the Class Bivalvia. He hypothesizes a possible evolutionary path to create the double retina system in Pectinidae and Laternulidae through the duplication of sensory structures on the pallial folds. Zieger and Meyer-Rochow review the variation of cephalic gastropod eyes, concentrating on pulmonate species, which are the best-studied eyes in gastropods. They discuss eye anatomy, differences in retinal design, and the visual capabilities of different optical components. Finally, they describe the ultrastructure of "additional" or "accessory" eyes associated with cephalic eyes in several lineages. These data provide hints of the function of these structures and indicate behavioral experiments to test these hypotheses.

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* From the symposium "Molluscan models: Advancing our understanding of the eye" presented at the World Congress of Malacology, held from 15 to 20 July 2007 in Antwerp, Belgium. Co-sponsored by the National Science Foundation and the American Malacological Society.

A comparative approach also can be used to examine changes in development, not just morphological endpoints. For example, even though most gastropods have eyes, loss of eyes occurs in some eyed lineages. Often eye loss is associated with dark environments, such as abyssal depths or caves, but little is known of when or how eye loss occurs. Averbuj and Penchaszadeh show that eyes are present in the "eyeless" genus *Buccinanops* (d'Orbigny, 1841) (Caenogastropoda: Neogastropoda) during the encapsulated larval stage. What happens to these cephalic eyes post-hatching is unknown, but why have eyes in non-motile larvae? Do other "eyeless" species have eyes as larvae and lose those eyes after the veliger stage? Studying these and other "eyeless" taxa may provide data on the evolutionary constraints of development on morphology. This is a promising area for future research.

Another way to study the eye is to examine differences among the various components that comprise the organ. Eyes are not just single, irreducible entities but they contain levels of biological complexity nested in a hierarchical fashion (*e.g.*, Serb and Oakley 2005). Therefore, the eye can be subdivided into components, or modules, such as genetic networks (*i.e.*, *Pax6* network), photoreceptor cell types, crystallin proteins that make up the lens, photo-transduction pathways that convert light into a chemical signal, and the eye itself as a morphological structure. Several authors focus on specific eye modules.

One module consists of crystallin proteins, which form the lens in both vertebrate and invertebrate eyes. Evidence indicates that these proteins initially performed biochemical functions unrelated to vision and were later recruited for optical purposes during the evolution of the eye lens (Cvekl and Piatigorsky 1996). In the symposium, Piatigorsky discusses the origin and evolution of lens crystallins in cephalopod and bivalve molluscs via processes of gene recruitment, gene sharing, and gene duplication.

Other eye components are the light-sensitive cells, photoreceptors, which are ubiquitous in animal eyes. Salvini-Plawen presents an interesting hypothesis on the evolution of the major classes of animal photoreceptors. He suggests that despite the structural differentiation of ciliary *versus* rhabdomeric photoreceptor cells, these cells are not distinct classes, but the result of ontological changes of a single cell type. Support for his hypothesis includes a comprehensive treatment of molluscan photoreceptor diversity. Wilkens examines the physiology of photoreceptors in bivalves—specifically, how do photoreceptor cells respond to light and how is this information processed outside of the eye? Based on physiological and behavioral work, he describes differences between species and among photoreceptor cell types within a single eye. Finally, he hypothesizes the functions of bivalve eyes.

In addition to these published papers, other symposium participants presented work on a range of topics. Eernisse reviewed the sensory system of chitons (Polyplacophora) and discussed how the recent appearance of chiton ocelli may have evolved in parallel in two phylogenetically distant lineages. Kelly and Robles (Kelly *et al.* 2008) added to the work of Gray *et al.* to identify a translational regulation mechanism for cytoskeleton proteins that have differential expression in light- versus dark-adapted octopus eyes. Speiser and Johnsen (2008) experimentally show that scallops use visual cues to adjust feeding behavior relative to the movement and size of particles suspended in the water.

I would like to thank the participants and the audience members who made the symposium an interactive experience and generated much discussion. I would also like to thank Thierry Backeljau and his team for organizing the UNITAS Antwerp meeting and Paula Mikkelsen for her support of the symposium. The symposium was funded by a grant from the National Science Foundation (NSF) (DEB 0614153) and the American Malacological Society (AMS).

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Toward developing models to study the disease, ecology, and evolution of the eye in Mollusca*

Jeanne M. Serb

Department of Ecology, Evolution and Organismal Biology, 253 Bessey Hall, Iowa State University, Ames, Iowa 50011, U.S.A., serb@iastate.edu

Abstract: Several invertebrate systems have been developed to study various aspects of the eye and eye disease including *Drosophila*, *Planaria*, *Platynereis*, and most recently, the cubozoan jellyfish *Tripedalia*; however, molluscs, the second largest metazoan phylum, so far have been underrepresented in eye research. This is surprising as mollusc systems offer opportunities to study visual processes that may be altered by disease, vision physiology, development of the visual system, behavior, and evolution. Malacologists have labored for over a century as morphologists, systematists, physiologists, and ecologists in order to understand the structural and functional diversity in molluscs at all levels of biological organization. Yet, malacologists have had little opportunity to interact with researchers whose interests are restricted to the biology and development of eyes as model systems as they tend not to publish in the same journals or attend the same meetings. In an effort to highlight the advantages of molluscan eyes as a model system and encourage greater collaboration among researchers, I provide an overview of molluscan eye research from these two perspectives: eye researchers whose interests involve the development, physiology, and disease of the eye and malacologists who study the complete organism in its natural environment. I discuss the developmental and genetic information available for molluscan eyes and the need to place this work in an evolutionary perspective. Finally, I discuss how synergy between these two groups will advance eye research, broaden research in both fields, and aid in developing new molluscan models for eye research.

Key words: retina, photoreceptor, opsin, Pax6

Traditional model systems to study eyes

There is a great diversity of metazoans, but research on developmental processes has largely focused on a small number of "representative" species. The traditional "big six" model organisms used in developmental biology are the roundworm *Caenorhabditis elegans*, the fly *Drosophila melanogaster*, the zebrafish *Danio rerio*, the African clawed frog *Xenopus laevis*, the chicken *Gallus gallus*, and the mouse *Mus musculus*. These species were developed as model organisms because they are amenable to experimental and/or genetic manipulation and possess life history characteristics suitable for life in the laboratory, *i.e.*, they are easy to obtain, breed readily, and are fecund. Research focused on these six model animals has resulted in large-scale genome sequencing efforts, and complete or near complete inventories of genes and high-resolution genome maps are now available for all six species (Waterson *et al.* 2002).

Of the two traditional invertebrate models, *Caenorhabditis elegans* and *Drosophila melanogaster*, only *Drosophila* possesses eyes. The *Drosophila* compound eye has been an outstanding model system to study many general developmental processes including cell fate specification, cell division, growth, and death (Pappu and Mardon 2004). In addition to exploring cellular biology, researchers have determined the molecular basis of eye specification by genetically dissecting the fly eye to understand how it works. We have discovered how a group of multipotent cells (stem cells) can be converted to eye primordia during eye organogenesis and have identified the set of nuclear genes that regulate retinal specification. Understanding these genetic mechanisms involved in eye formation gives researchers crucial information on the origin of eye disease—which is when the genetic program goes wrong.

The *Pax6* paradigm

Comparative work with the *Drosophila* eye and vertebrate eye indicates that all eyes may share a similar developmental pathway in eye formation (Fig. 1). This has been referred to as the *eyeless*/*Pax6* paradigm (Donner and Maas 2004), which states that a single homologous genetic network regulates eye formation, regardless of eye type, across all metazoans, and the *Pax6* gene or its homologs are part of this regulatory gene network (Fig. 1A). There are three lines of evidence for this conclusion. First, the gene *eyeless* (*ey*) in *Drosophila* is homologous to the genes *Small eye* of mice and *Aniridia* of humans (Quiring *et al.* 1994). These two verte-

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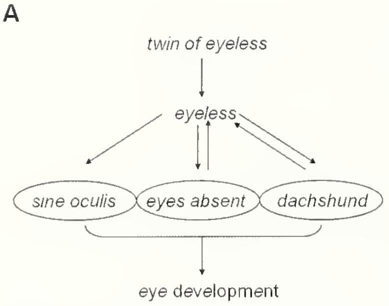

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Drosophila	Vertebrate
eyeless / twin of eyeless	Pax6 (9)
sine oculis	Six (6)
eyes absent	Eya (4)
dachshund	Dach (2)

Figure 1. A network of regulatory genes involved in eye formation conserved between *Drosophila* and vertebrates. A, The network of genes that regulates eye formation in *Drosophila*. Proteins from three genes in circles form biochemical complexes with each other in vertebrate models. B, List of vertebrate genes homologous to the *Drosophila* genes. Multiple vertebrate homologs, due to paralogous duplication, are indicated by numbers in parentheses (Relaix and Buckingham 1999, Donner and Maas 2004).

brate genes (*Small eye*, *Aniridia*) are collectively referred to as *Pax6*. This homology of *ey* and *Pax6* suggests that eye formation is controlled by a similar genetic mechanism in insects and vertebrates, despite large differences in eye morphology and development. Second, the *eyeless* gene has been shown to initiate eye formation. For example, when the *eyeless* gene in fly is mis-expressed (turned on in the wrong place at the wrong time, developmentally) eyes can be induced to form in wing, antennae, or leg primordia (Halder *et al.* 1995). Third, expression of *Pax6* gene copies from other species, including mice, squid, arrow-worm, and planaria, can also induce eye formation in *Drosophila* (Halder *et al.* 1995, Tomarev *et al.* 1997). The result of this work in *Drosophila* and vertebrates illustrates that there is a deep homology and conservation of eye genes in metazoans. This has lead some researchers (*e.g.*, Gehring and Ikeo 1999) to describe *Pax6* and its homologs as the "master control" gene for eye development in metazoans. We now know that this is an oversimplification of the system, and in fact, a number of other genes [*i.e.*, *eyes absent* (Bonini *et al.* 1997), *dachshund* (Shen and Mardon 1997), *sine oculis* (Pignoni *et al.* 1997) (Fig. 1B)] in addition to *Pax6*, are able to induce ectopic eye expression. Rather than a single gene, the *Pax6* paradigm really refers to a homologous genetic pathway that controls eye development across metazoans.

Molluscs as "non-traditional" model organisms for studying the eye

Despite the monumental advances in understanding eye development using traditional model organisms, it is important to include non-model systems in eye research. Broad comparative studies with many animal examples identify general evolutionary processes. Further, studying the eyes in multiple species expands our understanding of variation among eye types, how similar visual tasks many be performed under different conditions, how permutations at the structural level affect performance, and how gene and gene pathways evolve to create new phenotypes and subsequently, new functions.

In addition to the "traditional" *Drosophila* model, several other invertebrate organisms have been used to study the eye and eye disease including the flatworm *Planaria* (Saló and Baguñá 2002), the annelid *Platynereis* (Arendt *et al.* 2002), and most recently, the cubozoan jellyfish *Tripedalia* (Piatigorsky and Kozmik 2004, Nilsson *et al.* 2005). Work on planarian worms has provided a better understanding of eye formation, development, disease, and evolution of genetic networks (Pineda *et al.* 2000, Cebria *et al.* 2002), while *Platynereis* and *Tripedalia* have been used primarily as evolutionary models. *Platynereis* and *Tripedalia* models have broadened the evolutionary perspective of how eyes have evolved and what the ancestral eye condition may have been for Urbilateria (Arendt and Wittbrodt 2001, Arendt 2003, Piatigorsky 2003). Ultimately, these "non-traditional" models have given evolutionary depth to eye research by expanding work from the traditional model organism.

However, the second largest metazoan phylum, the Mollusca, has been underrepresented in eye research during the molecular age (post-*Pax6* paradigm) and has been underutilized in the study of developmental processes of the eye. This is surprising, as molluscan systems have shown potential for study of basic visual processes, physiology of vision, development of the visual system, and evolution. For example, past work (Robles *et al.* 1995, Torres *et al.* 1997) has shown that cytoskeletal organization of photoreceptor cells is regulated by the state of light- and dark- adaptation in cephalopod eyes. It is known that some disease states in the human retina, such as macular degeneration, affect cytoskeletal development and organization (Eckmiller 2004). Therefore, studies on cephalopod photoreceptors could lead to a better understanding of the role of the cytoskeleton in photoreceptor function and provide clues that link its organization to retinal disease. The goals of this paper are to: (1) provide an overview of the advantages of working with molluscan eyes; (2) describe the eye types found in molluscs; and (3) discuss the future directions of the field of eye research using molluscan models.

What is an "eye"?

An eye is a structure that can measure the amount of light (intensity) and compare light intensity from multiple directions (Land and Nilsson 2002). Therefore, eyes supply information of light distribution in the environment. Essentially, vision uses the principles of geometry to focus light (optics) and chemistry to transform light energy into chemical signals. A nerve center, such as the brain or cerebral ganglia, then interprets these signals. Therefore, the ability of an organism to 'see', referred to as spatial vision, is the interpretation of the origin and direction of light, intensity, and contrast in the organism's environment. These attributes of light are the basis of pictorial information as resolved images.

The simplest way to produce spatial vision is to have series of light sensitive cells (photoreceptors) shielded on one side by dark pigment cells (Fig. 2). Pigment cells are often arranged in a cup-shape, which prevents all of the photoreceptor cells from detecting light from the exact same angular direction at the exact same time. Adding more photoreceptor cells and increasing the depth of the cup-shaped eye (Fig. 3A) increases sensitivity to the direction of light and refines the image (Land and Nilsson 2002).

In metazoans, there are two major types of photoreceptor cells, which use two different means of increasing the cell's surface area to better capture light (Table 1). Ciliary photoreceptors have an expansion of the ciliary membrane, while rhabdomeric (or microvillar) photoreceptors have an array of villi (microvilli) on the cell membrane (Eakin 1979). Each photoreceptor type is associated with specific families of photo-pigment molecules, such as opsin (r-opsin in rhabdomeric vs. c-opsin in ciliary cells), and proteins of the photo-transduction cascade which convert light energy into a membrane potential, an electrochemical signal (Arendt 2003, Nilsson 2004) (Table 1). Photoreceptors can either be excited by light and transmit information on light intensity and direction, or light may inhibit photoreceptor response so that neurons are activated only when light is terminated—resulting in a response to shadow (Land 1968). The eye can be specialized further with the addition of lenses (lenticular eyes; Fig. 3B) and corneas. These structures are found in some eyes to help focus light and the image onto the aligned photoreceptors that make up the retina.

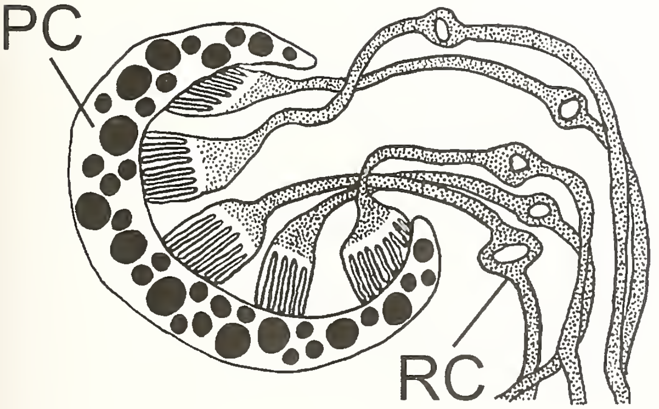

Figure 2. The simplest eye that produces spatial vision. Pigment cells (PC) are arranged in a cup-shape, which prevents all of the photoreceptor cells (RC) from detecting light from the exact same angular direction at the exact same time. Redrawn from Land and Nilsson (2006).

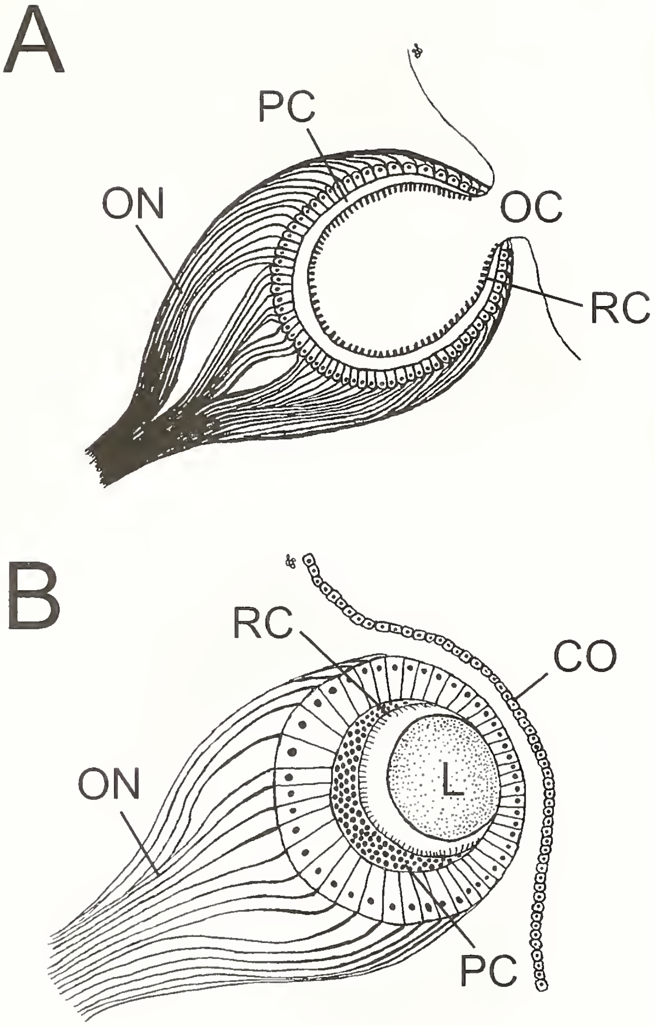

Figure 3. Two common eye types in molluscs. A, Open pit eye does not have a lens or cornea and is open to the environment (opening to optical cup; OC). B, Closed lenticular (lens) eye has both lens (L) and cornea (CO). Both eyes have optic nerves (ON), photoreceptor cells (RC), and pigment cells (PC).

Eye performance varies greatly among organisms and the specific eye structure that they possess. Performance of the eye can by summarized by two components: resolution and sensitivity (Land 1981, see Land and Nilsson 2002 for expanded explanation). Resolution is the precision with

Table 1. Characteristics of two photoreceptor cell types, rhabdomeric and ciliary. Information from Arendt (2003) and Nilsson (2004).

Photoreceptor cell type	Rhabdomeric	Ciliary
Membrane expansion	Microvillar	Ciliary
Photopigment molecule	Gq (G-protein)	Gi or Go[a] (G-protein)
Analogous proteins of phototransduction cascade	PLC (phospholipase enzyme)	PDE (phosphodiesterase)
	Arrestin-β	Arrestin-α
	rk 2, 3 (rhodopsin kinase)	rk 1 (rhodopsin kinase)
Membrane potential	Depolarizing	Hyperpolarizing

[a] Kojima *et al.* (1997).

which the eye can separate light according to its direction of origin and is directly related to the eye's ability to discriminate fine detail. Resolution depends on the number and spacing of photoreceptor cells in the retina. Sensitivity is the ability of the eye to capture enough light for photoreceptors to produce a usable neural signal, thus fully utilizing the potential resolution. Sensitivity can be increased by enlarging the aperture of the optical system, such as increasing the size of the pupil, or increasing photoreceptor diameter. However, increasing photoreceptor diameter decreases the number of photoreceptors in the retina, subsequently reducing the resolution of the eye. In general, a larger eye, with more photoreceptors and a large aperture, has both better resolution and sensitivity.

In molluscs, the placement of eyes is highly variable and may depend on both the function and the development of particular regions. In lineages such as gastropods and cephalopods, a pair of eyes is located on a well-developed head region; these are referred to as cephalic eyes. Other lineages with reduced head regions, such as polyplacophorans and bivalves, have many non-cephalic eyes. Some polyplacophoran species have eyes on exposed dorsal regions, while some lineages of bivalves have eyes on mantle tissue near siphons or along the valves.

The molluscan eye has many functions. Eyes are used for visually orienting the animal in its environment. For example, both bivalves and gastropods use visual cues: in bivalves, *Argopecten irradians* (Lamarck, 1819) appears to orient swimming behavior based on visual information (Hamilton and Koch 1996) while *Littorina* (Linnaeus, 1758) uses visual cues to discriminate between environments or objects (Evans 1961, Hamilton and Winter 1982). Eyes are also used to detect visual motion. For example, behavioral experiments in ark clams (Arcidae) *Arca noae* (Linnaeus, 1758) (Patten 1886), *Arca zebra* (Swainson, 1833), *Barbatia cancellaria* (Lamarck, 1819), and *Anadara notabilis* (Roding, 1798) (Nilsson 1994) suggest that the great number of eyes found on these species are used to detect motion, rather than responding to shadows. Ability to form an image also varies among molluscs. Coleoid cephalopods are probably best known for their excellent perception of images and ability to visual discriminate (review in Messenger 1981); perhaps lesser known is the wide degree of visual capabilities found among gastropods (Messenger 1981, Zieger and Meyer-Rochow 2008). Finally, it should be noted that mollusc eyes may also be important for migratory behaviors in pelagic and benthic species (Hamilton 1985).

Advantages and limitations to studying the molluscan eye

There are many advantages to working with a molluscan model to study the eye. First, molluscs provide an evolutionary perspective in eye research with a diversity of eye phenotypes within a single lineage rather than a comparison between the traditional model organisms that belong to disparate animal phyla. Although the *Drosophila* and vertebrate models have demonstrated the deep homology in eye genetics, we still lack a detailed understanding of what changes occur in these genetic networks that create the vast variation in morphology. With closely related mollusc lineages that possess different eye morphologies, we can tease apart changes at the gene level that alter phenotypes. Second, molluscs possess an array of visual adaptations found within a single species (*e.g.*, Groeger *et al.* 2006) or among closely-related species (*e.g.*, Kano and Kase 2002). These adaptations can be experimentally treated as "mutant" phenotypes, demonstrating the vast array of possible morphologies and providing a study system to examine specific genotypes that relate to phenotype. Third, molluscs are a powerful example of multiple, independently derived, image-forming eyes found across three (possibly four) classes. Multiple origins of complex structures allow researchers to test questions of gene or genetic network recruitment, an important mechanism that appears to have wide application to alter developmental processes resulting in novel phenotypes. Fourth, in molluscs a variety of eye types are expressed at different life stages within a single individual. This system can be used to test hypotheses of how duplication of orthologous or paralogous eye structures may have played a role in morphological and functional diversification of animal eyes (Oakley 2003, Friedrich 2006). Fifth, molluscs have the ability to regenerate their eyes, a reactivation of developmental processes in an adult organism to restore missing tissues (Butcher 1930, Bever and Borgens 1988, Bobkova *et al.* 2004b). Regeneration occurs across many different animal lineages, including amphibians, molluscs, crustaceans, planaria, and cnidarians. Comparative studies to understand how vastly different organisms are able to regenerate organs will identify both differences and similarities in the genetic process, mechanisms, and elements, such as multipotent progenitor cells or pluripotent "stem cells." By determining the genetic mechanisms of regeneration across metazoans, we may be able to apply components of these processes to human medicine. Molluscs offer a unique system to study how optic nerves are repaired during regeneration, and may be a useful model to develop regeneration therapies. Finally, the large camera-like eyes of the coleoid cephalopods are morphologically and physiologically similar to a vertebrate eye, but offer unique research advantages and opportunities to study eyes without the disadvantages or constraints of working with a vertebrate system.

Despite these advantages to using the molluscan eye to study eye development and evolution, there are some limitations to our current knowledge of molluscan eyes. First, most information of eyes in molluscs comes from only a handful of species (Hamilton 1991). Second, there have been few comparative studies within lineages or within species (but see Bobkova *et al.* 2004a, Gál *et al.* 2004, Speiser and Johnsen 2008a), so we may be underestimating the degree of variation in eye structure and function. Since visual systems may change during the life time of an organism, due to metamorphosis or changes in environment/habitat (Groeger *et al.* 2005, 2006), additional work is needed to understand these fine and coarse modifications. Third, although we have identified eye or eye-like structures in many mollusc lineages, we know little about the function of these structures (see discussion on sensory structures of the Polyplacophora below) and how these structures may be important to the life cycle or ecology of the organism.

Types of molluscan eyes

The number of eye types in the Mollusca mirrors the incredible diversity in body plans in the phylum. Of the seven mollusc lineages, four (Polyplacophora, Bivalvia, Gastropoda, Cephalopoda) contain species with eyes that minimally consist of photoreceptors (arranged as a retina), pigment cells, and a lens. These four lineages represent the greatest biological diversity within the Mollusca, encompassing over 98% of recognized mollusc species (Ruppert and Barnes 1994). Below is a brief overview of the eye types found in these four molluscan classes. I chose to focus on only a few examples in each lineage and refer the reader to many excellent reviews where appropriate.

Polyplacophora

While most studies on the optics and fine-structure of the molluscan eye focus on the bivalves, gastropods, and cephalopods, the polyplacophorans have a unique system of photoreceptors and eye-like structures. There are no cephalic eyes in polyplacophoran species. Instead, chitons have developed three types of photoreceptors in the shell, a condition unique to Mollusca. Shell eyes, or aesthetes, are imbedded in the tegmentum that covers the shell plates (Blumrich 1891). Aesthetes are found in all chitons and may function as simple photoreceptors to meditate light-response behavior (Boyle 1977, see Knorre 1925 for alternative functions), and most likely do not provide visual information. Extra-pigmentary ocelli (Moseley 1885, Nowikoff 1907) and intrapigmentary ocelli (Nowikoff 1909) are restricted to few lineages in the family Chitonidae (Boyle 1977) and are believed to be photoreceptors capable of determining direction and intensity of light. Unlike the aesthetes, both ocelli types have lenses, a vitreous area, and a cup of retinal cells with microvillous rhabdomes (Boyle 1969b), the components necessary for spatial vision. Like other non-cephalic eyes in molluscs, the ocelli are highly repetitive structures, where a single individual of *Onithochiton neglectus* (Rochebrune, 1881) can have 411 to 1,472 ocelli in rows along all shell valves (Boyle 1969b). A detailed account of the orientation, patterning, and cellular structure of the externally pigmented eye is provided by Boyle (1969a, 1977). Reviews on chiton sensory organs can be found in Charles (1966), Boyle (1977), Messenger (1981), Kaas and van Belle (1985), and Serb and Eernisse (2008).

There is much work to be done to understand the function and ability of the different types of sensory organs in chitons. Optics, physiology, and function of the three sensory organs have not been examined in any detail. Thus, it is not known if the two ocelli types function as "eyes" with the capability of spatial vision. Further, there has been no recent work on visually mediated behavior. Until we have this basic knowledge of ocelli in chitons, polyplacophorans cannot used as effective models.

Bivalvia

There is an incredible amount of morphological variation in eyes of the Bivalvia (review in Morton 2001). Most bivalve eyes are not cephalic as bivalves do not have a distinct head. Instead, the majority of eyes in adults are found along the edge of the mantle, referred to as pallial eyes. This position of the eye appears to be a type of ectopic expression, and many species that possess pallial eyes have a large number of serially repeated eye structures along the mantle.

Two of the most complex and unusual eye types in bivalves are found in the ark clams (Arcoida) and the scallops (Pectinidae). The first description of eyes in ark clams was Will (1844). Subsequent work by Patten (1886) and Nilsson (1994) refined the description of eye structure and examined eye function with visual behavioral experiments. Members of the Arcoida have two eye types: (1) a multifaceted compound eye which is similar in structure to the arthropod compound eye, but appears to be an independent origin of this eye type (Charles 1966, Nilsson and Kebler 2007) and (2) a simple pigment cup, or invaginate, eye (Fig. 3A). In contrast, Patten (1886, 1887) gives a description of three eye types: pseudo-lenticulate (groups of ommatidia, over which a cuticula is thickened to form a lens-like body), invaginate, and faceted (compound) eyes. However, recent treatments recognize only two eye types (Waller 1980, Nilsson 1994). Both eye types are found on the first outer mantle fold (Waller 1980), but the anterior-posterior patterning of the eyes varies across species and has been hypothesized to be related to the degree of light exposure of that portion of mantle edge (Waller 1980, Nilsson 1994). The eyes in Arcoida are highly repetitive structures, where a single individual may possess 200-300 compound eyes. This provides the animal overlapping visual coverage, which may improve sensitivity to the visual signal.

Based on measurements of eye performance calculated by eye anatomy, Nilsson (1994) suggests that the pallial eyes of ark clams function as optical "burglar alarms." According to this interpretation, these eyes are used to detect visual motion, rather than relying on a simple shadow response that can be accomplished by simple photoreceptors. The result is that the animal can respond to moving objects that do not cast a shadow. Although this means that ark clams have *spatial resolution* (ability to detect objects), it does not mean that they have the ability to visually reconstruct their environment, or *spatial vision* (Nilsson 1994). To date, the electrophysiology or neurophysiology of the ark clam eye has not been examined.

One of the best-known molluscan eye types is the mirror eye of the scallop (Pectinidae) and its close allies (Limidae, Spondylidae) (Patten 1886, Dakin 1910, 1928), where the image is not formed by the lens, but by reflection from the hemisphereical tapetum (argentea) that lines the back of the eye behind a double retina (description of optics in Land 1984). It has been demonstrated mathematically that the image forms on the distal retina, composed of ciliary photoreceptors (Land 1966a), and both physiological and behavioral experiments corroborate this finding (Patten 1886, Land 1966b). Pectinids respond to (1) an overall distribution of brightness in the environment, which determines the direction of swimming behavior via the proximal retina; local changes in (2) light intensity by shadow or (3) movements in the optical environment are involved in defensive responses via the distal retina (original description in Buddenbrock and Moller-Racke 1953, summary in Land 1968). So while the distal retina is used for focusing an image and detection of movement, the proximal retina response is to absolute levels of light intensity (Land 1966b). The two retinas function independently from one another with opposing responses to light (hyperpolarizing in distal retina vs. depolarizing in proximal retina) (Hartline 1938, Land 1966b, Gorman and McReynolds 1969, Gomez and Nasi 1994), are composed of different photoreceptor cell types (cilary vs. rhabdomeric) (Miller 1958), and use distinct phototransduction cascades (Kojima *et al.* 1997) (see Table 1).

Much work has been done on the scallop eye including recent work on optics (Land 1965, 1966a), comparative anatomy (Morton 2000, 2001 and references therein, Speiser and Johnsen 2008a), electrophysiology (Gorman and McReynolds 1969, Gomez and Nasi 1994), neurophysiology (Spagnola and Wilkens 1983, Wilkens 2006), visual-mediated behavior (Wilkens and Ache 1977, Hamilton and Koch 1996, Wilkens 2006, Speiser and Johnsen 2008b), phototransduction (Kojima *et al.* 1997), and lens formation and protein evolution (Carosa *et al.* 2002, Piatigorsky 2008).

The ark clam and scallop utilize two very different eye morphologies to obtain spatial information from their environments. Although the general structure of these eyes is not comparable, the functions may be quite similar. Yet, there has been much discussion of why a relatively sedentary organism, like a bivalve, would need such complex eyes and so many of them. Regardless of the specific function of bivalve pallial eyes, the large number found in scallops and ark clams strongly suggest that vision or visually mediated behaviors are extremely important to these species.

Gastropoda

Except for a few genera, most gastropods have a pair of cephalic eyes. Eye placement varies among gastropod groups, and the eye can be located at the base of cephalic tentacles, on the tips of retractable tentacles that can withdraw the eye, or on short stalks. Gastropod eyes range from open pits (Fig. 3A) to closed vesicles with or without lenses. The majority of gastropod eyes are of the closed lenticular type (Fig. 3B), composed of cornea, lens, vitreous body, and a cup-shaped retina (but see heteropods below). The retina can have multiple photoreceptor types (Table 1); however, the majority of photoreceptors near the lens are microvillous R cells that form rhabdomeres. Other photoreceptor cells (*e.g.*, H cells, basal retinal neurons -BRN) are ciliary (Chase 2002). Across species, there is considerable variation in retinal composition (number of cells, photoreceptor density, organization of photoreceptors) (Hamilton 1991, Chase 2002), but the functional significance of these differences largely is unknown and unexplored. Generally, gastropod eyes appear to have several functions including: mediating phototaxic behavior and locomotion, regulating daily and seasonal activities, and, in some species, visual detection of forms. However, the extent to which gastropod eyes have spatial vision is still under investigation (Zieger and Meyer-Rochow 2008) and will probably vary greatly among species.

There are several unique structures in the gastropod sensory system. The "accessory retina" (Smith 1906) is found in some gastropod lineages (*e.g.*, Limacidae), which may be involved in infrared detection (Newell and Newell 1968). Dorsal eyes appear in species of the marine slug *Onchidium* Buchanan, 1800. These eyes are on papillae projecting off the dorsum of the animal (Hirasaka 1922) and are composed of ciliary photoreceptors that may create a "reasonable image" (Land 1968). See detailed descriptions in Katagiri *et al.* (2002) and references therein. Probably the most sophisticated and unique eye in the gastropods is the scanning lenticular eye of pelagic heteropods. The retina is not cup-shaped but forms a long strip of 3-6 cells in width, resulting in a very narrow field of view and contains several photoreceptor types that are unlike ciliary or rhabdomeric receptors found in cephalic eyes of other molluscs (Land 1984). These eyes move in a systematic scanning motion, which may be used to detect stationary objects (Land 1982). Further work on the function of these unusual eyes is needed. For more information on gastropod eye diversity, there are several excellent and comprehensive reviews (Charles 1966, Messenger 1981, Chase 2002, Zieger and Meyer-Rochow 2008).

Cephalopoda

Vision in cephalopods is quite different than the visual information available to most other molluscs as reflected by the sophisticated eye types found in this lineage. There are two well-known cephalopod eye types, the pin-hole eye type of nautiloids and the camera-type eye (Fig. 4) in coleoid (internal shelled) cephalopods. Eyes of *Nautilus* Linnaeus, 1758 are unusual as they are open to the environment, have no cornea or lens, and appear to function like a pin-hole camera (Messenger 1981). Although it would appear that this eye is rather unsophisticated, many other features suggest that the eye has an effective visual system. The *Nautilus* eye is large, comparable in size to the more elaborate camera-type of the coleoids, and has an adjustable pupil (Hurley *et al.* 1978). Having a large eye with a small aperture improves spatial resolution of the eye, until light capture is limited. In fact, calculations by Land (1981) suggest that the resolving power of a nearly closed pupil of *Nautilus* is on par with a typical insect eye. In the retina, density of closely packed rhabdomeric photoreceptors has been conservatively estimated at an order of magnitude higher than any other non-cephalopod mollusc (Barber and Wright 1969, Messenger 1981), again implying good capabilities for image resolution (but see Land 1981). In addition, the *Nautilus* eye possesses an ocular-motor reflex which compensates for movement of the animal and allows for the stabilization of images on the retina. This type of reflex is found only in animals that are able to detect motion and form, thereby suggesting *Nautilus* has these capabilities (Land 1981).

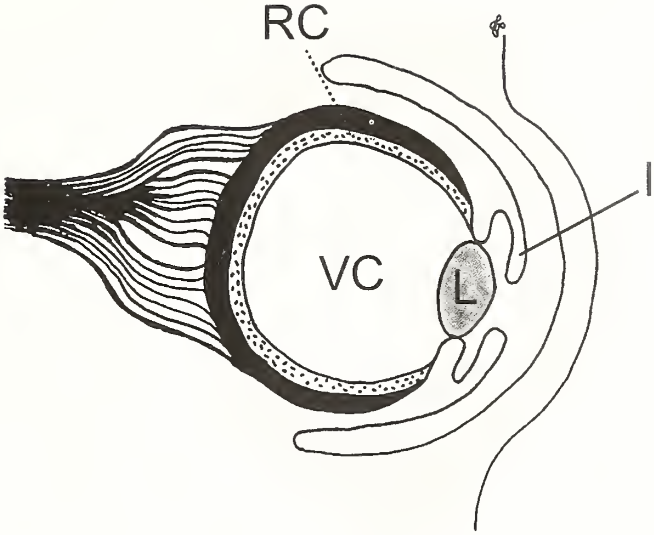

Figure 4. Camera-type eye of coleoid cephalopods has an iris (I), nearly circular lens (L), vitreous cavity (VC), and photoreceptor (RC) and pigment cells that form the retina. Redrawn from Zuker (1994).

Although these eye properties are important in spatial vision, the function of the *Nautilus* eye is still speculative. It has been suggested that the *Nautilus* may use its eyes to stabilize itself under strong oceanic currents, help navigate during diurnal vertical migrations, or identify potential food sources (Muntz 1991); however, little is known of the *Nautilus* in its natural condition and these hypotheses remain untested. Many other questions about the fine structure of *Nautilus* eye also remain unanswered.

In contrast to the eye of the *Nautilus*, considerable information exists on the eye and visual capabilities of coleoid cephalopods due to detailed studies of optics, neurophysiology, retinal organization, and extensive behavioral experiments. Since these animals rely on vision for prey capture, predator avoidance, and intra-specific communication (Budelmann 1996, Hanlon and Messenger 1996, Muntz 1999), they possess excellent perception and visual acuity (Messenger 1981).

Coleolid cephalopods have rhabdomeric (microvillous) photoreceptors in a camera-type eye that optically functions in a similar manner to the vertebrate eye, making the cephalopod eye a famous example of convergent evolution (Packard 1972; however, see Serb and Eernisse (2008) and references within for alternative views). Specifically, the cephalopod eye resembles an all-rod elasmobranch eye with similar optics, speed, sensitivity, and resolution (Packard 1972). Similarly, cephalopods have an iris, nearly circular lens, vitreous cavity, and photoreceptor cells that form the retina (Fig. 4); however, the photoreceptors in the cephalopod eye are rhabdomeric, not ciliary as in vertebrate eyes (Young 1962). Reviews on the similarities and differences of these eyes can be found in Packard (1972), Messenger (1981), Land (1984), Nicol (1989), Nilsson (1996), and Land and Nilsson (2002).

There is a large body of literature on the cephalopod retina. We have a good understanding of the retinal structure in both *Octopus* Cuvier, 1797 (for example, see early work in Babuchin 1864, Hensen 1865, summarized in Young 1962, Yamamoto *et al.* 1965, Messenger 1981) and squid (*Loligo* Lamarck, 1798) (Cohen 1973a, 1973b). Because the cephalopod retina is structurally simple, comprised of only a few cell types, it has been a favorite model system for the study of comparative physiology and photoreceptive mechanisms (Yamamoto *et al.* 1965). Other work on the cephalopod retina has focused on light/dark adaptation—how the eye acclimates to changes in light levels in the environment (Young 1963a). Work on cephalopods has identified important sub-cellular alternations to the shape of photoreceptor cells and movements of cytoskeleton and photopigments within these cells in response to changes in light intensity (Robles *et al.* 1995, Marinez *et al.* 2000, Gray *et al.* 2008). These studies have important application to understanding human eye disease. Another important question with medical applications is, how does the retina organize? While Meister (1972) provides a chronology of cell patterning and differentiation of the squid retina based on light microscopy, the next step is exploring the developmental regulation of the retina and how retinal cell fate is determined. This is a wide-open area for future research and will provide data to compliment work in mouse and other vertebrate models (Livesey and Cepko 2001, Zaghloul *et al.* 2005). These comparative studies of convergent structures will be an important contribution to both developmental and evolutionary biology.

Within coleolid cephalopods, there is both interspecific and intraspecific variation in their eyes. Some of these differences occur in the shape of the eye, which may deviate from spherical to telescopic (*Amphitretus* Hoyle, 1885), stalked (*Bathothauma* Chun, 1906), or asymmetrical (*Histioteuthis* Orbigny, 1841) eyes (Chun 1913, Nixon and Young 2003 and references therein). Composition of the eye may also vary. For example, the eyes of *Cirrothauma murrayi* (Eschricht, 1836) are simple open cups, lacking lens, iris, or ciliary body—the muscle and choroid surrounding the eye typically found in other coleolid cephalopods (Chun 1913). Patterning, size, and density of the rhabdoms in the retina vary among species (Young 1963b), and these traits appear to be correlated with the behavior and pupil shape (discussion in Messenger 1981). Absorbance and transparency of lenses can differ among species found a different depths (Denton 1960, Denton and Warren 1968, Sweeney *et al.* 2007b) as well as within a single species (Denton and Warren 1968). Finally, retinal sensitivity can vary during ontogeny. Recent work on the cuttlefish *Sepia officinalis* Linnaeus, 1758 indicates that aspects of the eye change during growth, including spectral sensitivity, light and contrast sensitivity, and visual acuity (Groeger *et al.* 2005, 2006).

The visual abilities in coleolid cephalopods have been explored more extensively than any other molluscan group (reviewed in Messenger 1981). Cephalopods display excellent perception and are able to discriminate between different shapes, but it appears that they are color blind (Messenger 1981, Mathger *et al.* 2006). So how do these cephalopods create and control their camouflage to imitate chromatically rich environments without color vision (Hanlon 2007)? An interesting solution has been suggested by Shashar and Cronin (1996). They propose that polarized vision may provide visual information to detect and recognize objects analogous to color vision systems. Polarized light sensitivity has been identified in many cephalopods (Moody and Parriss 1960, Jander *et al.* 1963, Tasaki and Karita 1966), suggesting its importance in the organism's ecology (*e.g.*, Waterman 1981), but the function of this sensitivity needs to be tested further.

FUTURE DIRECTIONS

Choosing a molluscan model

Considerations

To further advance eye research in molluscs, a directed and combined effort to develop one or several model species is needed. Some considerations in choosing a model organism should include its life history traits, the availability of the nuclear genome sequence of the target species or a closely related species, and a strong foundation of research in the eye system of that species (Bolker 1995, Slack 2006, Jenner and Wills 2007). For experimental work, it will be necessary to maintain the model organism for a period of time in the laboratory. Development and implementation of a new molluscan model species will depend on both species characteristics and laboratory considerations. For example, to maximize the number of possible laboratory experiments per year, it is optimal if both adults as well as embryos are available year-long by either culturing the species in the laboratory or collecting samples from wild populations. In addition, housing costs for the species must be considered, especially if a colony needs to be maintained. Larger species will require more laboratory space, and marine species may be more challenging, especially if the species filter-feeds. For eye research, placement and size of the organ is also important. Eyes of potential model organisms must be easily accessible in the adult or at specific development stages, if experimental manipulation or explants are necessary. For questions concerning genetic processes, the model organism would need to be a laboratory-cultured species with quick embryonic development that reaches sexual maturity in a short period of time. In these cases, small animals would be preferred to house many individuals and to keep maintenance cost down. However, it has been pointed out that species selection based on rapid developmental rate and small body size may introduce bias such as developmental and genomic constraints or maternal influence (Bolker 1995, but see Jenner and Wills 2007 for the opposing view).

For nearly all eye research, an organism with a complete genome sequence would be advantageous. First, the availability of the complete nuclear sequence of a model organism gives the researcher a complete inventory of all genes in that organism. Second, identifying and isolating homologous genes in the new model organism becomes almost trivial compared to the laborious method of cloning homologous genes in a new species. Third, all members of a gene family could be identified in advance of the experiment. These data are essential to interpretation of gene function and its manipulation. Currently, Genbank of the National Center of Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) lists three genome projects of mollusc species that are in progress or are being assembled and annotated. These include the freshwater snail *Biomphalaria glabrata* (Say, 1818) (Gastropoda: Basommatophora)—in progress (Washington University); the sea hare *Aplysia californica* Cooper, 1863 (Gastropoda: Opisthobranchia) (Broad Institute)—in assembly; and the marine clam *Spisula solidissima* Dillwyn, 1817 (Bivalvia: Veneroida) (Marine Biological Laboratory)—in progress. Another genome that is currently being annotated is the limpet *Lottia gigantea* Sowerby, 1834 (Gastropoda: Patellogastropoda) available at the Joint Genome Institute (JGI; http://genome.jgi-psf.org/Lotgi1/Lotgi1.home.html).

Finally, successful development of a molluscan model organism would benefit from a body of research already conducted on that species. Previous work on such topics as optics and visual behavior may direct the types of questions or direct which organism may be most appropriate for the project. That being said, development of a new model organism is a time consuming process as well as a large financial commitment for genomic resources and laboratory setup. Only taxa that have multiple uses can realistically be considered.

Candidate model species

There are several molluscan species that meet several of the above criteria and make strong model organism candidates. *Aplysia californica* might be considered the highest priority for molluscan eye researchers. This species has been the "workhorse" for both physiology and neurobiology, and there is a large body of literature on the physiology, neurology (neurobiology, neural processes), photoreception, and visual-mediated behavior (Kandel 1979 and references therein). *Aplysia californica* has a pair of small (300-600 μm) cephalic eyes at the base of the posterior tentacles (rhinophores). The eye is a closed chamber with a large spheroid lens. The retina, which nearly surrounds the lens, appears to have both rhabdomeric and ciliary photoreceptors that interdigitate to form the rhabdomere (Jacklet *et al.* 1972). Based on the close proximity of lens to retina, the *A. californica* eye does not appear to have good spatial vision, but the eyes respond to light in three different ways (Jacklet 1969, 1973). This demonstrates that the two photoreceptor types respond differently to light, like the scallop, making this an interesting system. Keeping and culturing *Aplysia* in laboratory has been somewhat standardized (Kandel 1979), and the National Institute of Health (NIH) and University of Miami run a large-scale mariculture facility, the *Aplysia* Resource Facility, that can provide specimens from known genetic lines for researchers (http://www.rsmas.miami.edu/groups/sea-hares/), making availability of specimens a non-issue. In addition, the *Aplysia* genome has been sequenced and is being assembled.

A bivalve model for eye research might be a scallop species. There is a large body of literature on the scallop eye (see references above). Scallops are commercial species being cultured in aquaculture facilities for the global market, so it should be an easy transition to develop a facility for research. This also presents an opportunity to collaborate with aquaculture researchers to develop genomic tools and resources, such as sequencing the scallop genome.

Unlike *Aplysia* and scallops, cephalopods have large eyes making them easy to work with and manipulate. Several coleoid cephalopod species would make good model organisms to study the eye due to their availability. Three small species (*Sepia officinalis*, *Sepia pharaonis* Ehrenberg, 1831, and *Euprymna scolopes* Berry, 1913) are laboratory-cultured by the National Resource Center for Cephalopods (NRCC), which is funded by NIH's National Center for Research Resources and Texas Institute of Oceanography. As squids have been a popular model organism, many texts and protocols are available for eye and nervous system work (*e.g.*, Gilbert *et al.* 1990, Meinertzhagen 1990, Saibil 1990). Currently, an EST (expressed sequence tag) library for the eye is available for a related species, *Octopus vulgaris* Cuvier, 1797 (Ogura *et al.* 2004), which provides a list of genes expressed in a specific tissue (the eye) at a particular developmental stage (adult). This genomic resource could be applied to other cephalopod species as a list of candidate genes or as a starting point to isolate specific genes or gene families in cephalopod eyes. In addition, one of the transcription factors initiating eye organogenesis, the *Pax6* gene, has been isolated from the squid *E. scolopes* and there are developmental data on the role of *Pax6* in eye, brain, and sensory organ development (Tomarev *et al.* 1997). Detailed studies on the development of the eye and central nervous system in several species (Marthy 1973, Shigeno *et al.* 2001) and development and structure of the lens (West *et al.* 1995, Sweeney *et al.* 2007a) are available. A large body of literature exists for cephalopod ecology and how these organisms adapt to environmental conditions (Boyle and Rodhouse 2005 and references therein). Finally, cephalopods may be the best molluscan model for medical research because their eye structure and function are similar to the human eye.

Molluscan models in evolutionary biology

Molluscs are an excellent group for the study of evolutionary biology because, as a group, they possess a diverse set of eye phenotypes that range in complexity. For example, within a single lineage like Gastropoda, eye phenotypes range from simple pit eyes to complex lenticular eyes. Across Mollusca, nearly every eye type is represented as well as many unique phenotypes. Among metazoans, molluscan eyes will provide data for a more comprehensive view of eye evolution, rather than relying on a few model organisms found in widespread and distant phyla. In particular, molluscan eyes are a compelling case of multiple, independently derived, image-forming organs. Within the eye there are various levels of homology to examine, including the level of the gene and genetic network (*e.g.*, *Pax6* pathway), cell (*e.g.*, photoreceptor), or tissue type [lens protein evolution, (Carosa *et al.* 2002, Piatigorsky 2008)]. Below are some examples of evolutionary topics that can be addressed with molluscan eye models.

Testing the Pax6 *paradigm*

In a recent paper by Donner and Maas (2004), the genetic pathways used to create an eye were compared in *Drosophila* and vertebrates. The authors found that while all genes in the *Drosophila Pax6* pathway are expressed in the vertebrate eye during development, the functions and relationships of these homologous genes within their respective pathways have not been strictly conserved. This being the case, Donner and Maas (2004) argue that *Drosophila* is still a valuable study model and may be used to guide research on vertebrate eye development. They conclude (p. 750) that when the pathway is not strictly maintained between vertebrates and invertebrates, this indicates that "the particular role that the genetic [pathway] . . . is *either not relevant*, or *not sufficient, to meet the complexity of the vertebrate* [eye]" (my italics). One interpretation of these results is that conservation of genetic pathways between lineages will decrease as eye complexity increases, and eye types diverge, in one lineage. We can test this assertion in two ways using molluscan models. First would be to deal with a major shortcoming with the Donner and Maas' (2004) hypothesis, namely that their comparison was between two completely different eye types: the compound eye of *Drosophila* and the camera-type eye in vertebrates. A more appropriate test might be a comparison of two camera-type eyes—in cephalopods and in vertebrates—that are similar in function but vary in their degree of retinal complexity. Second, the hypothesis could further be tested by examining changes in the *Pax6* pathways and the resulting phenotype of the eye within a single molluscan lineage, such as gastropods or bivalves, that have multiple eye types ranging from simple photoreceptor eyespots to more complex lenticular eyes.

Using the molluscan eyes to examine evolution

The eye has long been a target of anti-evolutionists as an example of "irreducible complexity" (Behe 1996) The idea is that certain biological systems, such as eyes, are too complex to have evolved from simpler, less complete, prototypes and that these structures are too complex to have arisen from chance mutations (Hall and Hall 1975, Johnson 1991, Oakland and Matrisciana 1991, Behe 1996). Anti-evolutionists often cite a single sentence from Darwin's *Origin of Species* to demonstrate his own doubt in the ability of evolutionary forces to create the eye:

> "To suppose that the eye [in all of its complexity] . . . could have been formed by natural selection, seems, I freely confess, absurd in the highest possible degree" (Darwin 1859: 186).

Less often is Darwin's (1859: 186) next sentence cited, which states:

> "Yet reason tells me, that if *numerous gradations* from a perfect and complex eye to one very imperfect and simple, each grade being useful to its possessor, *can be shown to exist*; if further, the eye does vary ever so slightly, and the variations be inherited, which is certainly the case; and if any variation or modification in the organ be ever useful to an animal under changing conditions of life, then the difficulty of believing that a perfect and complex eye could be formed by natural selection, though insuperable by our imagination, can hardly be considered real" (my italics).

Molluscs, with their diversity of eye types, offer examples of an "intermediate" eye types and the group is often cited as a counter-argument to anti-evolutionists. The range of eyes include the eye spot, pigment cup (Fig. 3A), pin hole camera eye (*Nautilus*), lenticular eye (*i.e.*, *Strombus* Linné, 1758; Fig. 3B), and the camera eye (*Octopus*; Fig. 4). However, it has not been demonstrated that a single lineage contains a plausible series of intermediate eye-designs to examine the gradient hypothesis. Gastropods may be a good group to test the gradient hypothesis as there are several lineages within the gastropods that contain variation in eye complexity. Once a species phylogeny has been constructed and eye types characterized on the tree, ancestral states of eye complexity can be estimated. In addition, the time to transition from one eye structure to another could be calculated. This would be an excellent test of Nilsson and Pelger's (1994) estimated time (about 0.5 million years) needed to evolve a lenticular eye from a simple photoreceptor patch.

Photoreceptor evolution

Both rhabdomeric and ciliary photoreceptors are found in two (*Aplysia* and *Pecten*) of three of potential molluscan model organisms, which is useful for testing current views of photoreceptor evolution. In a recent paper by Plachetzki *et al.* (2005), the authors argue for a common origin and subsequent divergence of photoreceptor cells in an early metazoan ancestor based on two lines of evidence. First, both rhabdomeric and ciliary photoreceptors have been found to coexist in many lineages, including vertebrates (Panda *et al.* 2005) and annelids (Arendt *et al.* 2004). These data are part of a growing body of evidence that is in contrast to previous hypotheses, where rhabdomeric photoreceptors are found mostly in invertebrates and ciliary photoreceptors generally occur in vertebrates (Eakin 1979, 1982; however, see Vanfleteren 1982 for a differing view). Second, rhabdomeric and ciliary photoreceptors can be identified by specific genetic signatures, which are gene expression specific to that particular cell type, such as opsin, *rx*, and atonal genes. However, molecular data to support the hypothesis of Plachetzki *et al.* (2005) are limited to a few taxa (*e.g.*, the annelid *Platynereis*, the cubozoan *Tripedalia*, and the mouse). Sequence data from opsin and phototransduction signaling proteins expressed in molluscs will provide additional tests for this new view of photoreceptor evolution.

Conclusions

The range of molluscan eyes provides a diverse array of structures and functions and represent an excellent system for investigating developmental processes. In order to reap the rewards of this system, malacologists will need to take an interdisciplinary approach. Although tools developed and used in traditional model systems can be successfully applied to mollusc eyes, those interested in studying the molluscan eye must familiarize themselves with much of the existing eye literature and have an understanding of traditional model species used to study the eye. Therefore, these researchers must be trained in other biological fields with a diverse set of methods. In particular, advances in genomic techniques will make mollusc species more accessible to studying the genetics of the eye and its processes. Ultimately, these advances and new husbandry practices will allow the development of new molluscan species as models to study the eye.

It is important that molluscs are included in eye research, in part because molluscan eyes have much to offer developmental and cell biology, physiology, evolution, and ecology. The phylum provides a diversity of eye structures that possess a multitude of functions. Many times these highly diverse eyes are found within a species, providing opportunities to examine the genetic underpinnings of phenotypic variation. For cell biology, molluscan models have been successfully used to study cytoskeleton growth, which could provide clues that link its organization to retinal disease. In developmental biology, gene expression studies in molluscan models may offer insight in the mechanisms of cell differentiation and cell fate. Finally, the addition of molluscan taxa to the study of the eye will fill an evolutionary gap in understanding eye development and evolution.

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Rho signaling mediates cytoskeletal re-arrangements in octopus photoreceptors*

Shaunté M. Gray, Shannon Kelly, and Laura J. Robles

Department of Biology, California State University, Dominguez Hills, 1000 East Victoria Street,
Carson, California 90747, U.S.A., lrobles@csudh.edu

Abstract: Light sensitive rhabdoms in the octopus retina increase in cross-sectional area in the dark and shrink in the light. Growth in the dark is due to the formation of microvilli in an avillar region of the photoreceptor cell membrane and lengthening of rhabdomere microvilli already present. Diminution in the light is the result of the disassembly and shortening of the same microvilli. Each microvillus contains an actin filament core that must be assembled or disassembled in the dark or light, respectively. To understand the regulation of the construction and breakdown of rhabdomere microvilli in the light and dark, we used centrifugation to separate the rhabdom membranes followed by Western blotting and Rho pull-down assays to investigate the role of Rho GTPases in this process. Western blotting showed a difference in the distribution of Rho in rhabdom membrane and supernatant fractions. In the light, Rho was mostly present in the supernatant but in the dark it was found in the fraction enriched with rhabdom membranes. Complementing these results, pull-down assays showed that Rho is activated in the dark but in the light, Rho is mostly inactive. We believe that in the dark, activated Rho binds to the rhabdom membrane and initiates signaling pathways, leading to growth of rhabdomere microvilli. In the light, Rho is present in the soluble fraction, is inactivated, and is likely bound to a Rho GDI. Receptors involved in the activation of Rho in the dark are undetermined and may involve rhodopsin or another membrane protein.

Key words: cytoskeleton, rhabdoms, Rho pull-down assay

The cytoskeleton, consisting of microtubules, microfilaments, and intermediate filaments, is a three-dimensional infrastructure within cells responsible for a myriad of functions, including cell movement, cytokinesis, and organization of the cytoplasm. At any particular point in the cell cycle, the cytoskeleton has a unique architecture, which can undergo rapid reorganization in response to environmental or internal signals. Microtubules and microfilaments, composed of tubulin and actin subunits, respectively, generally reorganize by the addition or subtraction of their respective protein subunits, leading to lengthening or shortening of the tubules or filaments and consequent changes in cell shape or movement (Maekawa *et al.* 1999). Intricate signaling cascades activated in response to environmental or internal cues trigger these changes in cytoskeletal organization and are regulated by the Ras superfamily of small GTPases, including the Rho family GTPases (Ridley 2001, Raftopoulou and Hall 2004).

The Rho family GTPases, which currently includes 22 members such as the well known Rho, Rac, and Cdc42, shuttle between an active GTP-bound state and an inactive GDP-bound state (Takai *et al.* 1995, 2001, Hall 1998, 2005, Ridley 2001, 2006, Wheeler and Ridley 2004). In the activated state, Rho GTPases interact with specific downstream kinases that affect the state of actin polymerization. Rho and Rac indirectly affect actin polymerization by targeting ROCK (Rho-associated serine-threonine protein kinases) or Pak 1 (p21-activated kinase) that in turn activate LIM kinase 1 or 2 (LIM motif containing kinase). LIM kinases phosphorylate and inactivate actin binding/filament severing proteins, such as cofilin, which leads to an increase in actin polymerization (Naruyima *et al.* 1997, Arber *et al.* 1998, Maekawa *et al.* 1999, Sumi *et al.* 1999, Ohashi *et al.* 2000, Ridley 2006).

Photoreceptors of vertebrate and invertebrate retinas contain cytoskeletons that reorganize in the light and dark and may be regulated by the Rho family of GTPases (Miller *et al.* 2005). This reorganization is necessary to achieve maximum absorption of light by the photoreceptors by either increasing the membrane area containing the photopigment rhodopsin or a mechanical re-orientation of the photoreceptors so the light sensitive area sits more advantageously in light path, such as occurs in teleosts (Ali 1975). In either case, the cytoskeleton is involved in membrane growth or re-orienting the cell but the mechanisms leading to these changes are not well understood in any photoreceptor.

The octopus retina can serve as a model system to dissect these mechanisms since the photoreceptors of octopus

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species are large and only two cell types are present in the retina, photoreceptors and supportive cells, facilitating microscopic and biochemical studies. In *Octopus bimaculoides* (Pickford and McConnaughey, 1949), distinct changes in photoreceptor shape occur in the light and dark and may be directly attributable to changes in the organization of the cytoskeleton. Torres *et al.* (1997) compared the relative cross-sectional area of light- and dark-adapted rhabdoms, the light sensitive region of the cell, and outer segment core cytoplasm and found that the rhabdoms of light-adapted photoreceptors are reduced in cross-sectional area when compared to those maintained in the dark. Electron microscopy showed that the rhabdomeric microvilli in light-adapted rhabdoms partially disappeared, leaving behind an avillar membrane connecting the microvilli on opposite sides of the rhabdoms. In the dark, this avillar membrane was replaced with additional microvilli making the rhabdoms larger.

The biochemical mechanisms leading to increased microvillar formation in the dark and diminution in the light are not well understood. Miller *et al.* (2005) studied the presence of Rho GTPases in octopus retinas. Immunoblot analyses of whole retinal extracts confirmed the presence of Rho in light- and dark-adapted retinas and confocal microscopy localized Rho in the rhabdoms and showed its co-localization with actin.

The presence of Rho in the rhabdom suggests that it is involved in a signaling transduction pathway leading to rhabdom changes in the light and dark. If Rho were activated in the dark, it could indirectly inactivate the filament severing protein cofilin leading to rhabdom growth. To test this hypothesis we have performed detailed immunoblot analyses on isolated rhabdom compartments and Rho-GTP pull-down assays on light- and dark-adapted octopus retina tissue. Our work will lead to a better understanding of mechanisms leading to retinal changes in the dark and light in octopus species which affect their ability to absorb light. Furthermore, understanding cytoskeletal dynamics are important for all species and may lead to understanding specific types of retinal degeneration in humans attributed to cytoskeletal protein mutations such as Usher syndrome type 1B (Weil *et al.* 1995).

MATERIALS AND METHODS

Eye structure

Eye tissue was prepared for microscopic observations according to previously published methods and immunostained with antibodies to rhodopsin (Robles *et al.* 1995). Micrographs were made using an Olympus Vanox or BX461 fluorescence microscope.

Rho immunoblots

Adult specimens of *Octopus bimaculoides* were dark- or light-adapted for 2-3 hours. Afterwards they were anesthetized on ice, whole eye cups removed, and the sclera and lenses discarded. To isolate the rhabdom compartment from the remaining retinal tissue, we used previously described centrifugation procedures (Robles *et al.* 1984). These centrifugation experiments were repeated five times using a total of 20 octopuses per lighting condition. Results were consistent throughout each experiment.

Equal amounts of total protein supernatant from the first centrifugation, crude membrane pellet, and final pellet were diluted 1:1 with Laemmli reducing buffer (Bio-Rad Laboratories, Inc., Hercules, California), boiled for 5 minutes, and electrophoresed on 12% sodium dodecylsulfate polyacrylamide gels (SDS-PAGE) at 100V for 2-3 hours (Laemmli 1970). The proteins were blotted onto polyvinyldifluoride (PVDF) or nitrocellulose membranes and incubated overnight in 2.5% blocking solution (non-fat dry milk and gelatin in phosphate buffer saline [137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.4mM KH_2PO_4 0.1% Tween-20] Bio-Rad). The membranes were incubated overnight with polyclonal rabbit anti-Rho (-A-B-C) (1:1000 and 1:500, Upstate Cell Signaling Solutions) either overnight at 4 °C or for one hour at room temperature, followed by incubation with AP-GAR secondary antibodies (1:3000, Bio-Rad) for two hours at room temperature. All antibodies were diluted in PBST-BSA (Bio-Rad). An Opti-4CN Substrate kit (Bio-Rad) was used for colorimetric band detection, and the Bio-Rad VersaDoc™ 3000 imaging system was used for molecular weight analyses. Quantitative analysis of Rho concentrations in dark- and light-adapted octopus retinal fractions was obtained via densitometric analysis using the Quantity One 1-D Analysis software on the VersaDoc™.

Rho activation

At the end of light- or dark-adaptation, the eye cups from one octopus in each lighting condition were dissected to use as controls. The remaining octopuses were moved to the opposite lighting condition and sacrificed at 5, 15, 30, 45, and 60 minute time points. The preparation of retinal lysates from the controls and the time points was carried out using a dry ice-ethanol bath to snap freeze the tissue. Whole eye cups were removed and placed into a Petri dish in the dry ice-ethanol bath. Retinal tissue was liberated from the eye cups and added to 0.5 mL of cell lysis buffer (50mM Tris-HCL pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 µg/mL each Aproptinin, Leupeptin, Pepstatin; 1mM Na_3VO_4; 1 mM NaF) and homogenized. Samples were clarified using centrifugation for 5 minutes, 4 °C at 8,000 rpm and Rho activation assayed using the Rho Activation Biochem Kit (Cytoskeleton, Inc.). Supernatants were collected and pellets were discarded. Positive and negative controls were processed by adding a 1:10 volume of loading buffer. A nonhydrolyzable form of GTP (GTPγS) was added to the mixture in a 1:100 volume to a final concentration of 200 μM. This positive control sample was incubated for 15 minutes at 30 °C. The reaction was stopped by transferring the tube to 4 °C and adding a 1:10 volume of stop buffer. The same processing was carried out for the negative control, substituting GDP for GTPγS.

The supernatants and controls were added to 60 μl aliquots of GST tagged Rhotekin-RBD beads. Each reaction tube was incubated for one hour at 4 °C with gentle agitation. Next, the supernatant was removed and the pellet was rinsed with 500 μL 1X lysis buffer. The beads were pelleted at 5,000 g for 3 minutes at 4 °C. The supernatant was removed again and the pellet washed in 500 μL 1X wash buffer. Again, the mixture was centrifuged at 5,000 g for 3 minutes at 4 °C. The pellet was resuspended in a 3:1 ratio with Laemmli reducing buffer, boiled 5 minutes, and subjected to 15% SDS-PAGE at 120V for 2 hours.

The proteins were blotted onto nitrocellulose membranes and incubated for 45 minutes to one hour in SuperBlock + 0.05% Tween (Pierce) with gentle agitation. The membranes were incubated for one hour at room temperature or overnight at 4 °C with RhoA monoclonal antibody (1:500, Cytoskeleton) and for one hour at room temperature with HRP-GAM secondary antibody (1:20,000-1:50.000, Pierce ImmunoPure Peroxidase Conjugated GAM IgG(H+L)) at room temperature. All antibodies were diluted in TBS-Tween. The Pierce SuperSignal West Pico Chemiluminescent Substrate Kit was used for chemiluminescent detection. All blots were developed and visualized with the Bio-Rad VersaDoc™ imaging system. Quantitative analysis of the RhoA activation blots was performed using the Quantity One 1-D Analysis software previously described.

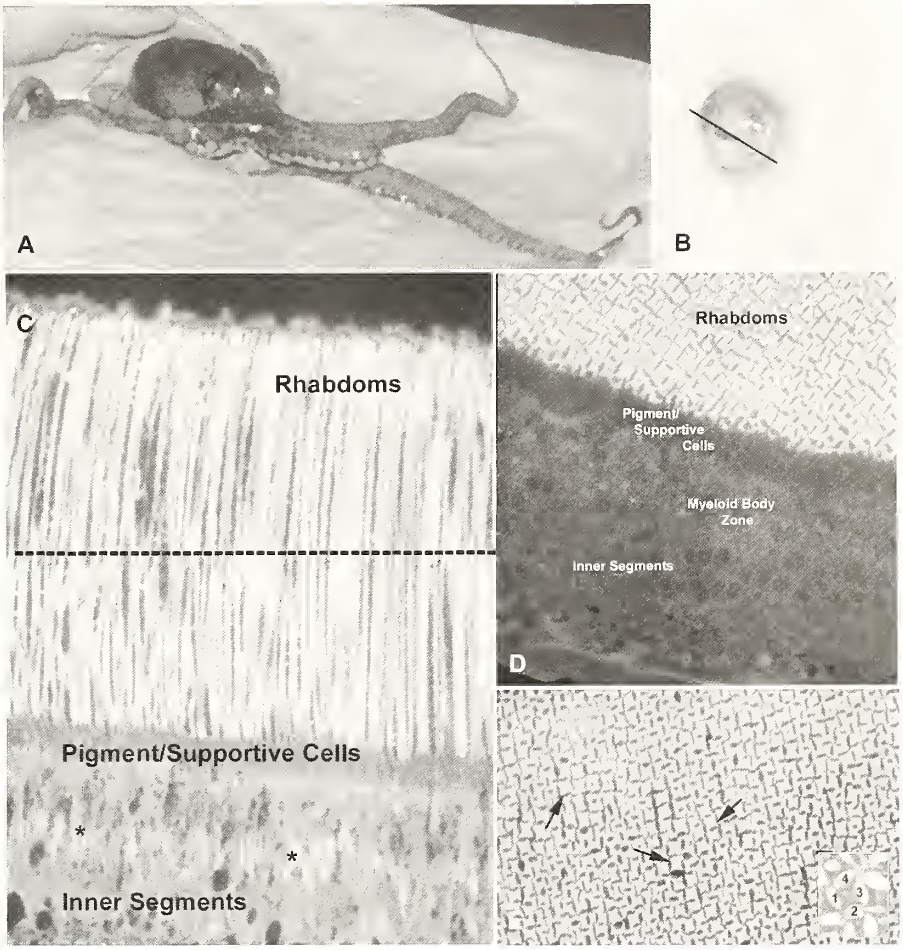

Figure 1. Retinal structure in *Octopus bimaculoides*. A, *O. bimaculoides*. B, Dissected eye from *O. bimaculoides*. Line through the iris marks the plane of sectioning, after removal of the iris and lens, to obtain retinal image shown in C. C, Longitudinal section through retina showing photoreceptor structure. The dotted line indicates plane of sectioning to obtain tangential to cross-sections through rhabdoms shown in D-E. D, Tangential section through retina to show details of rhabdom structure. E, Arrows highlight individual rhabdoms. In the inset, 1-4 denote the cytoplasm of four individual cells. Each side of the cells contributes one rhabdomere, composed of microvilli, which form the rhabdom.

RESULTS

Eye structure

The octopus eye is similar in its external appearance to other camera-like eyes. The slit-shaped iris permits light to pass through the spherical lens and focus on the retina at the back of the eye (Figs. 1A-E). The light sensitive retina of *Octopus bimaculoides*, as well as those of other octopus species, consists of a layer of photoreceptors and supportive cells (Fig. 1C). Optic nerves exit the photoreceptors at their base, form bundles, and extend to the optic ganglia where they synapse on ganglion cells. The photoreceptors span the

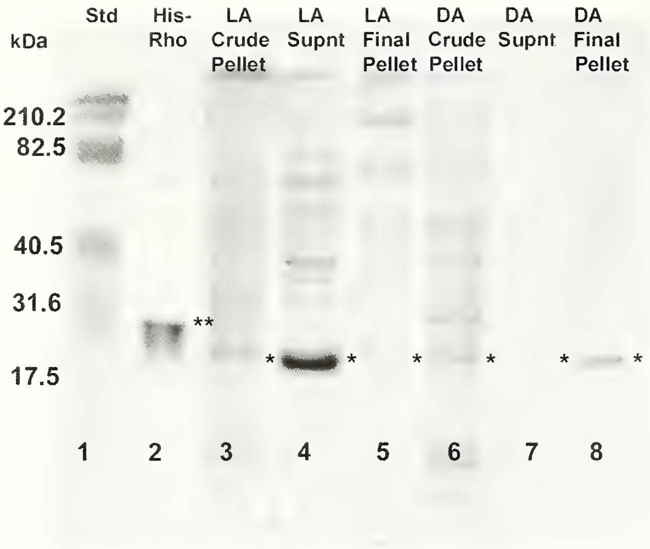

Figure 2. Western blot localizing Rho in purified light- and dark-adapted rhabdom membrane and supernatant fractions. Rho (*) is present in dark-adapted (DA) rhabdom membrane fractions (lanes 6 and 8) but not in the supernatant (lane 7). In the light-adapted (LA) supernatant fraction (lane 4), Rho is present while there is little or no detection of Rho in the LA rhabdom membrane fractions (lanes 3 and 5). The double asterisk denotes the His-tagged RhoA control protein (lane 2). The molecular weight sizes correspond to the fragments of the Kaleidoscope pre-stained standard (lane 1).

entire retina and are compartmentalized into the inner segments, a middle region which passes through the pigment/supportive cell layer, and the rhabdoms. The inner segments contain the biosynthetic machinery of the photoreceptor as well as organelles called myeloid bodies which store a second photopigment retinochrome. The photoreceptors narrow above the myeloid body region and pass between the supportive cells giving rise to the outer segments and rhabdoms. The outer segments consist of a cytoplasmic core and two sets of microvilli on opposite sides of the core which run from the base of the outer segments to their tips. These microvilli are called rhabdomeres, and rhabdomeres from four adjacent cells point toward each other to form the rhabdoms (Figs. 1D-E, see inset on 1E). The membrane of each rhabdomere microvillus contains rhodopsin and signal transduction proteins necessary to process the visual signal after light absorption by rhodopsin as well as a core of actin filaments and accessory proteins (Robles *et al.* 1995, De Velasco *et al.* 1999). As mentioned, the rhabdoms increase in size in the dark, by the addition and lengthening of the rhabdomere microvilli, and decrease in the light by the disassembly and shortening of the same microvilli (Torres *et al.* 1997).

Rho localization

Rhabdoms are easily separated from the rest of the retina using centrifugation techniques (see Materials and Methods). The rhabdom membranes can be separated from the outer segment cytoplasm using sucrose solutions and higher speed centrifugation. We obtained rhabdom membrane and supernatant (cytoplasm) fractions from light- and dark-adapted retinal homogenates of *Octopus bimaculoides* and performed immunoblot analyses on the rhabdom membrane enriched and supernatant fractions. We identified Rho GTPase in rhabdom membrane fractions and supernatants of light- and dark-adapted animals.

In homogenates obtained from light-adapted animals, faint Rho bands were visible in the crude and final enriched rhabdom membrane pellets, but a strong signal was detected in the supernatant after incubation with polyclonal anti-Rho-A-B-C (Fig. 2, lanes 3-5). In dark-adapted animals, bands are present in the crude and final rhabdom membrane pellet, but only a faint band is visible in the supernatant (Fig. 2, lanes 6-8). Control Rho (Fig. 2, lane 2, double asterisk) is His-tagged (Cytoskeleton, Inc.) and runs at 25 kDa compared to the endogenous Rho in our samples with a molecular weight of approximately 21 kDa. These results were consistent throughout repeated experiments. Background bands are likely due to non-specific binding and insufficient blocking of membrane before overnight incubation with anti-Rho to intensify the bands.

Quantitative analysis of Rho concentrations (adjusted percent volumes) on the blots reveal that Rho is approx. 10-fold more abundant in the light-adapted supernatant fractions (Fig. 3, lane 4) than in rhabdom membrane fractions (Fig. 3, lanes 3, 5) of light-adapted retinas. Rho appears

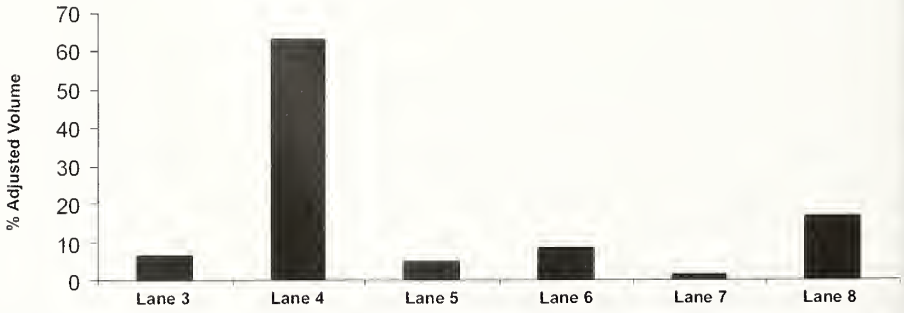

Figure 3. Quantitative analysis of membrane and supernatant fractions verifies the presence of Rho in the LA supernatant and DA membrane fraction. Lanes correspond to lanes in Fig. 2.

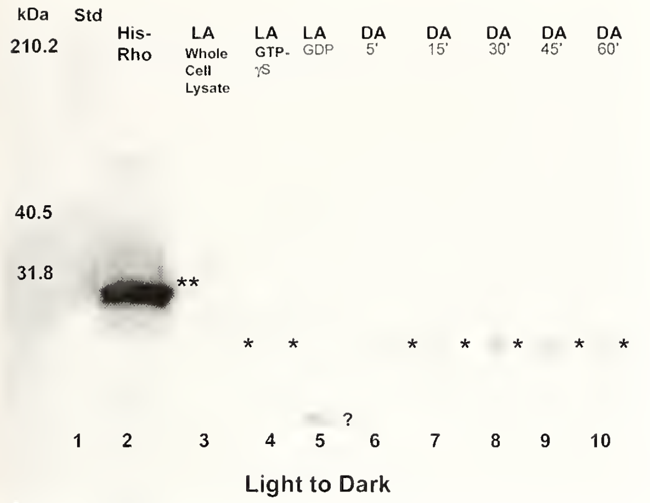

Figure 4. Rho pull-down assay showing Rho activation in light-adapted retinas that were moved to the dark and sampled at 5, 15, 30, 45, and 60-minute time points after the shift. Activated Rho, migrating between 20-22 kDa (*) is visible at all time points, most notably and strongly at 30 and 45 minutes. For comparison, Rho can be seen in the whole cell lysate (lane 3). There is little or no activation in the light-adapted GDP control (lane 5), but there is a strong band below 20 kDa (question mark) whose identity has not been determined. His-tagged RhoA control protein (**) migrating between 25-27 kDa is in lane 2.

most abundant in the rhabdom membrane fractions (Fig. 3, lanes 6, 8) than in the supernatant fractions (Fig. 3, lane 7) of dark-adapted retinas.

Rho activation

Rho pull-down assays were performed (Cytoskeleton, Inc.) to determine if Rho was activated in the light, dark, or in both lighting conditions and when the activation reaches its peak. After light- or dark-adaptation, octopuses were moved to the opposing lighting condition and sacrificed at 5, 15, 30, 45, and 60-minute time points. Pull-down assays performed at each time point confirmed the presence or absence of endogenous Rho-GTP with a molecular weight of approximately 20-21 kDa in dark to light or light to dark animals (Figs. 4, 6A-B). In addition to the His-tagged RhoA protein control (Cytoskeleton, Inc.) having a molecular weight of approx. 25-27 kDa, positive and negative controls included GTPγS and GDP loaded samples, respectively. Light- and dark-adapted GTPγS samples revealed the presence of activated RhoA while GDP-loaded samples showed little or no activated RhoA (Fig. 4, lanes 4, 5; Fig. 6A, lanes 3, 4). We also included whole cell lysates from light-adapted or dark-adapted animals to compare the activated RhoA signal with that of the total native RhoA (active and inactive) signal (Fig. 4, lane 3; Fig. 6, lanes 6, 7).

Light to dark

In animals that were light-adapted and then moved to the dark, Western blot analysis of pull-down products with anti-RhoA confirmed the presence of Rho-GTP at the 5, 15, 30, 45, and 60-minute time points, with peak activation at 30 and 45 minutes (Fig. 4, lanes 6-10). Rho is also present in the whole cell lysates and GTPγS control as expected (Fig. 4, lanes 3, 4). The GDP control (Fig. 4, lane 5) was negative except for a band at a very low molecular weight, possibly resulting from proteolysis of which the identity has not yet been determined.

Quantitative analysis of the light to dark blot revealed that the increase in activated RhoA is approx. 2-fold greater at 30 and 45 minutes after light-adapted retinas are moved to the dark (Fig. 5, lanes 6-10).

Dark to light

In animals that were dark-adapted and then moved to the light, Rho-GTP was detected faintly at 15 and 30 minutes but was more prominent after 45 minutes in the light (Fig. 6, lanes 9-13). Quantitative analysis of the dark to light blot revealed that RhoA is activated at much lower levels after 15, 30, and 45 minutes of light exposure (Fig. 7, lanes 9-13) when compared to retinas that were light-adapted and moved to the dark (Figs. 4, 5).

The increased background and non-specific binding seen (Fig. 4) are attributed to overnight incubation with

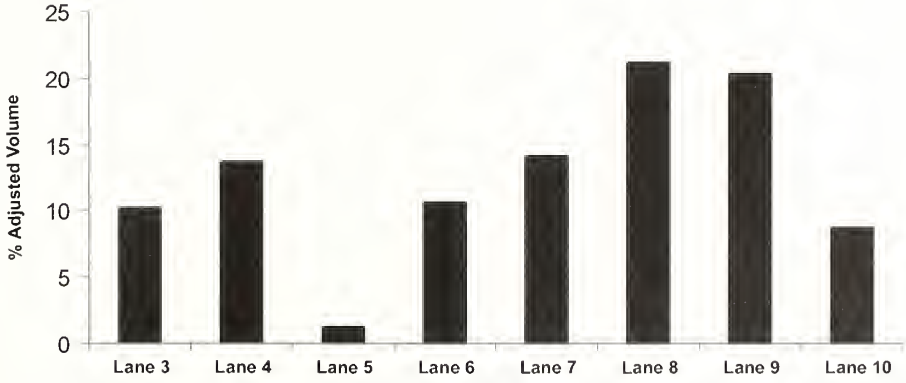

Figure 5. Quantitative analysis of the blot shown in Fig. 4 verifies the strong presence of activated Rho with the highest signal at 30 and 45 minutes.

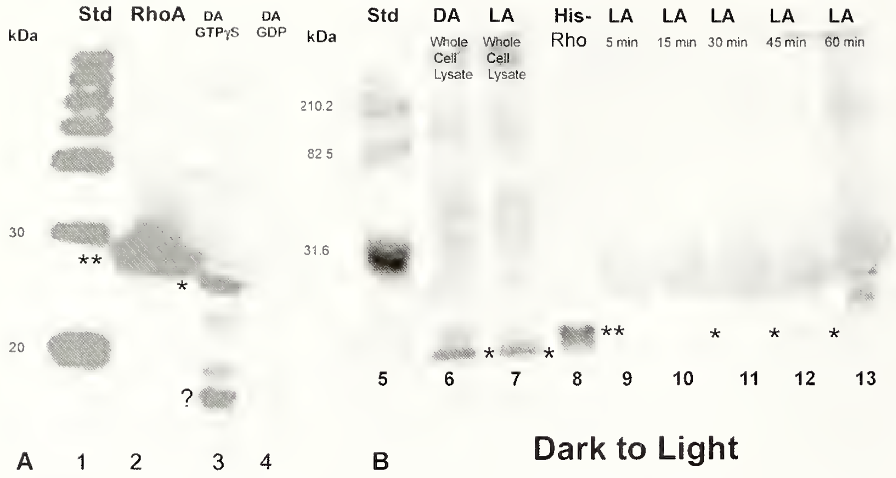

Figure 6. A, Western blot showing controls for the Rho pull-down assay for octopuses dark-adapted and then moved to the light (lanes 1-4). There is no activation in the dark-adapted GDP control (lane 4), but there is a strong band in the GTPγS control (lane 3, **). The strong band migrating below 20 kDa, is also present (*). Lane 2 contains the His-tagged RhoA control protein and standards are in lane 1. B, Western blot after Rho pull-down assay showing the detection of weak, residual Rho activation in dark-adapted retinas moved to the light and sampled at 5, 15, 30, 45, and 60 minutes after the shift. The detection of activated Rho (*) is visible at 15, 30, and 45 minutes after the shift to the light (lanes 10-12). Rho in whole cell lysates (activated and inactivated) is shown in lanes 6 and 7 and standards in lanes 1 and 5.

primary antibody at 4 °C to increase the Rho signal on the blots. Also, there is an increase in the number of bands above the targeted Rho protein band in Fig. 6B: the globular bands above 24-25 kDa are because of dimerization of the Rho protein with the Rhotekin beads used in the pull-down assay.

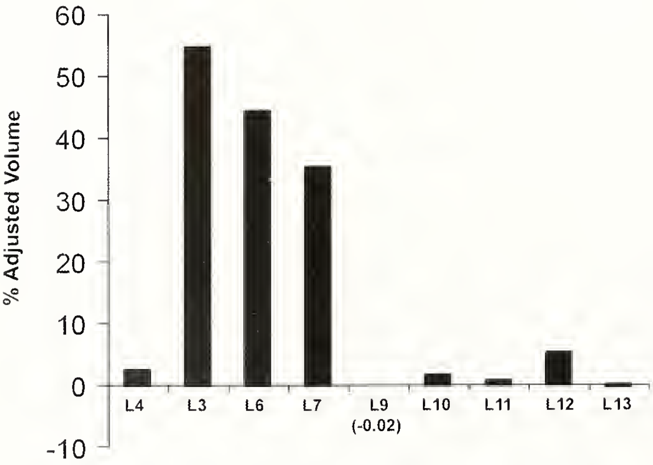

Figure 7. Quantitative analysis of blot in B verifies the weak, residual Rho in dark-adapted retinas moved to the light.

DISCUSSION

We previously reported the immunocytochemical localization of Rho in the rhabdom compartment of photoreceptors from *Octopus bimaculoides* (Miller *et al.* 2005). Rho, as well as actin, were present along the length of the rhabdomere and suggested that the Rho GTPases were candidates for the regulation of rhabdomere growth and diminution in the dark and light, respectively. Rho is present in the rhabdoms of light- and dark-adapted *O. bimaculoides* (Fig. 8 A-E). Using biochemical methods to localize Rho in the membrane fraction and pull-down assays to demonstrate Rho activation after light-dark-adaptation, the results reported here expand our earlier studies showing the presence of Rho in specific regions of the rhabdom and its activation in the dark or light.

The centrifugation techniques we used separate the rhabdom membranes from other retinal components and then further divide the compartment into membrane and soluble fractions (Robles *et al.* 1984). In tissue obtained from light-adapted animals, Western blotting showed that Rho was enriched in the supernatant and very little was found associated with the membranes. In the dark, Rho was mostly associated with the membrane fraction and little was found in the soluble fraction (Figs. 2-3).

Pull-down assays are used to confirm the presence or absence of a specific protein and are similar to an affinity-binding column. Specifically, Rho pull-down assays are designed to show the presence or absence of GTP-bound Rho in the tissue sample. Our assays on retinal homogenates from light- and dark-adapted *Octopus bimaculoides* showed that RhoA is mostly activated in membranes obtained from animals in the dark, with peak activation at 30-45 minutes, while RhoA activation was reduced in samples obtained from animals in the light.

Rhabdom cross-sectional areas increase in size in the dark and diminish in the light (Torres *et al.* 1997). This increase can be explained by either the addition of new microvilli or increase in size of microvilli already present. Addition or growth require membrane assembly and assembly of the actin core contained within each microvillus. The question is what factors initiate rhabdom growth. Our results are consistent with our hypothesis that in the light,

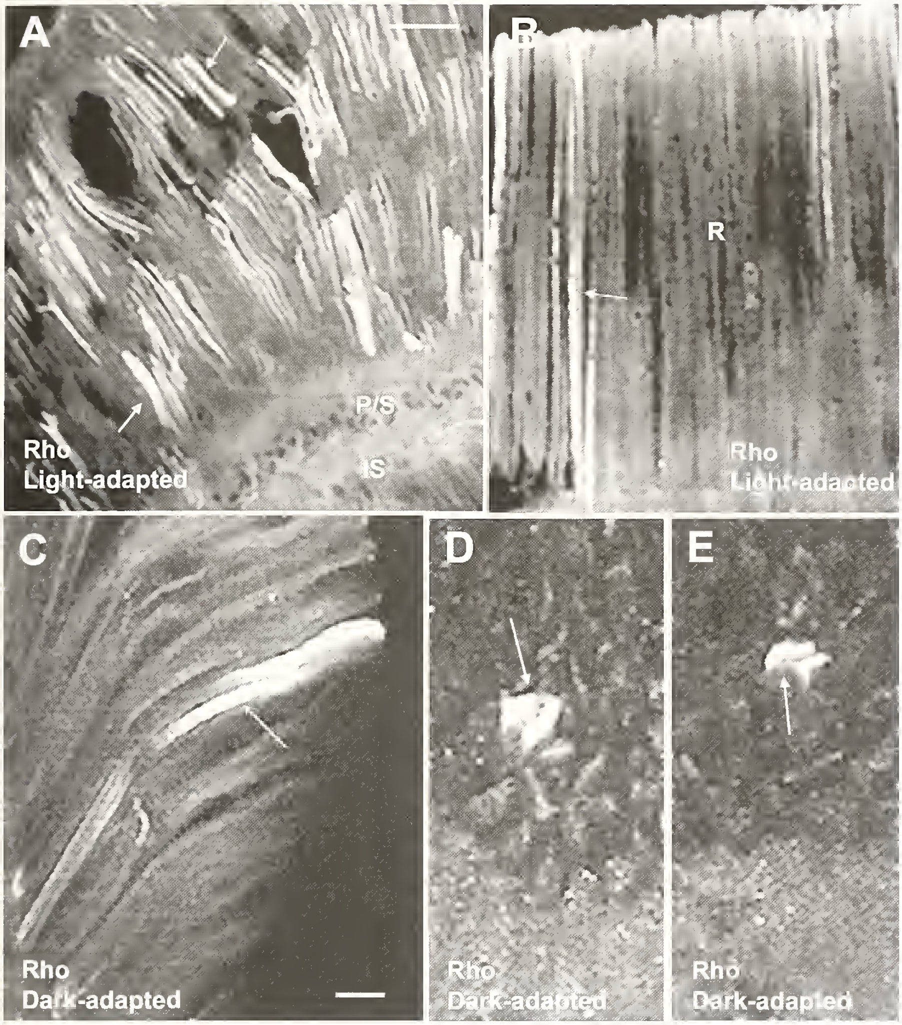

Figure 8. Rho localization of the retina of *Octopus bimaculoides.* A-C, Rho is present in the rhabdoms (arrows) of light- and dark-adapted photoreceptors. D-E, Cross-section through rhabdoms showing that Rho robustly labels some rhabdoms and not others. Scale bar = 100 µm A-B, 25 µm C-E. Reprinted with permission from Miller *et al.* (2005) *Visual Neuroscience* **22**: 295-304.

RhoA is sequestered by a GDI (Guanine Nucleotide Dissociation Inhibitor) present in the photoreceptor cytoplasm (supernatant fraction), preventing Rho from associating with the rhabdom membranes.

We postulate that in the light, Rho is sequestered in the cytoplasm bound to a GDI of which three are known in mammals (Fukumoto *et al.* 1990, Olofsson 1999, Dovas and Couchman 2005). We further postulate that rhodopsin inactivation in the dark leads to translocation of the GDI-Rho complex to the rhabdom membrane and release of Rho (Bokoch *et al.* 1994, Boukharov and Cohen 1998, Michaelson *et al.* 2001). GDIs form complexes with the Rac/Rho proteins, which are translocated when cells are activated (Seabra 1998, Kaibuchi *et al.* 1999). Moreover, GDIs are able to block GDP dissociation, inhibit protein phosphorylation, and increase the solubility of the Rac/Rho protein into the cytosol (Bokoch *et al.* 1994). Since we observed that Rho was mostly associated with the membrane fraction in the dark and that it is activated, we believe that Rho could then initiate a signaling pathway controlling acting filament assembly. Whether or not the signal to initiate the Rho signaling pathway comes from rhodopsin remains undetermined. More work is needed to better understand Rho signaling pathways governing cytoskeletal changes in the retina of *Octopus bimaculoides* and other species.

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Comparative morphology of the concave mirror eyes of scallops (Pectinoidea)*

Daniel I. Speiser[1] **and Sönke Johnsen**[2]

[1] Department of Biology, Duke University, Box 90338, Durham, North Carolina 27708, U.S.A., dis4@duke.edu

[2] Department of Biology, Duke University, Box 90338, Durham, North Carolina 27708, U.S.A., sjohnsen@duke.edu

Abstract: The unique, double-retina, concave mirror eyes of scallops are abundant along the valve mantle margins. Scallops have the most acute vision among the bivalve molluscs, but little is known about how eyes vary between scallop species. We examined eye morphology by immunofluorescent labeling and confocal microscopy and calculated optical resolution and sensitivity for the swimming scallops *Amusium balloti* (Bernardi, 1861), *Placopecten magellenicus* (Gmelin, 1791), *Argopecten irradians* (Lamarck, 1819), *Chlamys hastata* (Sowerby, 1842), and *Chlamys rubida* (Hinds, 1845) and the sessile scallops *Crassadoma gigantea* (Gray, 1825) and *Spondylus americanus* (Hermann, 1781). We found that eye morphology varied considerably between scallop species. The eyes of *A. balloti* and *P. magellenicus* had relatively large lenses and small gaps between the retinas and mirror, making them appear similar to those described previously for *Pecten maximus* (Linnaeus, 1758). In contrast, the other five species we examined had eyes with relatively small lenses and large gaps between the retinas and mirror. We also found evidence that swimming scallops may have better vision than non-swimmers. Swimming species had proximal retinas with inter-receptor angles between 1.0 ± 0.1 (*A. balloti*) and 2.7 ± 0.3° (*C. rubida*), while sessile species had proximal retinas with inter-receptor angles between 3.2 ± 0.2 (*C. gigantea*) and 4.5 ± 0.3° (*S. americanus*). Distal retina inter-receptor angles ranged from 1.7 ± 0.1 (*A. balloti*) to 2.8 ± 0.1° (*C. rubida*) for swimming species and from 3.0 ± 0.1 (*C. gigantea*) to 3.6 ± 0.2° (*S. americanus*) for sessile species, but did not appear to correlate as strongly with swimming ability as proximal retina inter-receptor angles did. Finally, we found that optical sensitivity differed between species, measuring from 3 ± 1 (*A. balloti*) to 21 ± 10 $\mu m^2 \cdot sr$ (*C. hastata*) for proximal retinas and from 2 ± 1 (*C. gigantea*) to 8 ± 5 $\mu m^2 \cdot sr$ (*C. hastata*) for distal retinas. These differences, however, did not appear to correlate with ecological factors such as a scallop species' swimming ability, preferred substrate type, or range of habitat depth. In light of these and previous findings, we hypothesize that scallop distal retinas may perform tasks of similar importance to all species, such as predator detection, and that proximal retinas may perform tasks more important to swimming species, such as those associated with the visual detection of preferred habitats.

Key words: vision, visual ecology, comparative morphology, invertebrate biology

Scallops have more acute vision than any other bivalve mollusc (Warrant and Nilsson 2006), but it has been argued that their eyes, like those of other bivalves, function merely as "burglar alarms" that trigger valve closure when large passing objects are detected (Nilsson 1994). Scallops are also notable for their ability, in most cases, to swim by a form of jet-propulsion (Cheng *et al.* 1996) and there is some indication that their swimming behavior may be visually influenced. For example, it appears that scallops are able to visually detect and swim towards preferred habitats (Buddenbrock and Moller-Racke 1953, Hamilton and Koch 1996). Arguments have been put forth, however, that scallops are unable to perform visual tasks of such complexity due to the limitations of their decentralized nervous system (Morton 2000). We suspect that these limitations may not be as severe as once thought, given recent findings that other animals with decentralized nervous systems, such as box jellyfish (Coates 2003) and sea urchins (Blevins and Johnsen 2004), use image formation to help guide movement. We therefore believe that the relationship between scallop vision and swimming behavior is one worth continued study.

If scallops are able to visually detect preferred habitats, as we hypothesize, it may be expected that swimming species have more acute vision than non-swimmers. Alternately, if scallops only use their eyes to detect predators, it is likely that little difference exists between the eyes of mobile and immobile species. Little is known about how optical resolution and sensitivity vary among scallop species, but it is thought that eye morphology is largely conserved within Pectinoidea (Dakin 1928, Morton 2001), a superfamily (Waller 2006) containing both scallops and spondylids (for brevity, we will refer to all members of Pectinoidea as "scallops" in this report). All scallops so far examined have eyes lined with a concave spherical mirror that reflects focused light onto a pair of retinas as well as a lens that is believed to help correct for spherical aberration caused by the mirror (Land 1965).

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To test our hypothesis, we examined eye morphology by immunofluorescent labeling and confocal microscopy in the swimming scallops *Amusium balloti* (Bernardi, 1861; Fig. 1), *Placopecten magellenicus* (Gmelin, 1791), *Argopecten irradians* (Lamarck, 1819), *Chlamys hastata* (Sowerby, 1842), and *Chlamys rubida* (Hinds, 1845) and the sessile scallops *Crassadoma gigantea* (Gray, 1825) and *Spondylus americanus* (a spondylid; Hermann, 1781). We calculated inter-receptor angle (a measure of optical resolution) and optical sensitivity for each species and explored the relationships between these calculations and ecological factors such as a scallop's swimming ability, preferred substrate type, and range of habitat depth.

MATERIALS AND METHODS

Specimen collection and fixation

Four specimens apiece of *Argopecten irradians* and *Placopecten magellenicus* were obtained from Beaufort, North Carolina, U.S.A. and Woods Hole, Massachusetts, U.S.A., respectively. Three specimens of *Spondylus americanus* were obtained from the Florida Keys (Florida, U.S.A.), a single specimen of *Amusium balloti* was obtained from Australia's Great Barrier Reef, and single specimens of *Chlamys hastata*, *Chlamys rubida*, and *Crassadoma gigantea* were obtained from Friday Harbor, Washington, U.S.A. Animals were anesthetized in a 3% $MgCl_2$ solution prior to dissection. Excised eyes were fixed in buffered 4% formaldehyde for between two and twelve hours and then washed three times in PBTw, a buffer solution containing the mild detergent Tween 20™. Samples were next rinsed three times in 70% ethanol and stored in 70% ethanol, except for *C. hastata*, *C. rubida*, and *C. gigantea* tissue, which was rinsed and stored in 100% methanol. All samples remained in alcohol for less than two months before measurements were taken. Except for *C. gigantea*, in which all eyes were of nearly equal size, all examined species had both large and small mantle eyes. Only large eyes were used for measurements. Eyes from the ventral (middle) section of the left valve mantle margin were used for measurements whenever possible.

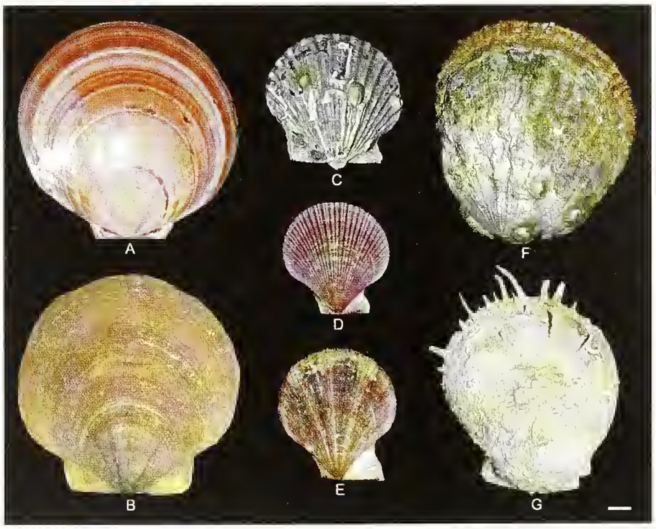

Figure 1. The left valves of the scallop species examined in this study. Pictured are the swimming scallops *Amusium balloti* (A), *Placopecten magellenicus* (B), *Argopecten irradians* (C), *Chlamys rubida* (D), and *Chlamys hastata* (E) and the sessile scallops *Crassadoma gigantea* (F) and *Spondylus americanus* (G). The scale bar represents 1 cm.

Sample preparation and measurements

For sectioning, fixed scallop eyes were cut in half with a scalpel blade. Eyes were only used for measurements if a clean, perpendicular cut was made through the center of the lens. Sectioned eyes were stained with fluorescently-labeled antibodies to alpha-tubulin, a microtubule protein, and Hoescht 33245, a DNA-binding fluorescent dye. Eyes were incubated in the anti-alpha-tubulin primary at 4 °C overnight and in an Alexa Flour 488 secondary for 4 hours at room temperature. Both the primary and secondary antibodies were diluted 1:500 in a blocking buffer which contained BSA powder and goat serum diluted in 1× PBS. After alpha-tubulin staining, 10 mg/mL Hoescht 33245 stock solution, diluted 1:100 in 1× PBS, was used to stain the eyes for five minutes. Stained eye sections were mounted in glycerol on standard microscope slides. Cover-slips were applied with modeling-clay feet so as not to disturb natural eye morphology. Eyes were mounted so that pupils and cover-slips were perpendicular. Images were obtained with the 10 or 20× objective of a Zeiss 510 LSM inverted confocal microscope housed in the Duke University Light Microscopy Core Facility. Illumination was provided by 405, 488, and 561 nm lasers. Images were processed on a Zeiss-built Fujitsu Siemens Intel Xeon CPU using Zeiss LSM 510 version 4.2 software.

Eye internal diameter, focal length (f), pupil diameter (D), photoreceptor spacing for distal (s_d) and proximal (s_p) retinas, and rhabdom length for the photoreceptors of the distal (l_d) and proximal (l_p) retinas were measured for each eye section. The image in a scallop eye is formed by the reflection of light off a concave spherical mirror (Land 1965), making focal length (f) equal to half the radius of mirror curvature (Halliday and Resnick 1988). We measured focal length by manually fitting circles to the mirror layer at the central section of each eye (the section in which the apparent curvature of the mirror matches its actual curvature), then calculating half the radius of the circle. Pupil diameter (D) was estimated from cornea diameter. Image stacks obtained with the microscope's 20× objective allowed us to study the morphology of individual photoreceptors from each eye's distal and proximal retina. Photoreceptors were distinguished from other cells by their strong staining by alpha-tubulin antibodies. Photoreceptor spacing (s) was calculated as the distance from the center of one photoreceptor's rhabdom to the center of the rhabdom of its nearest neighbor.

Calculations for optical resolution and sensitivity

We calculated inter-receptor angle for the distal ($\Delta\varphi_d$) and proximal ($\Delta\varphi_p$) retinas of each scallop eye section using the formulas:

$$\Delta\varphi_d = \tan^{-1}\left(\frac{s_d}{f}\right) \cong \frac{s_d}{f} \quad \text{and} \tag{1.1}$$

$$\Delta\varphi_p = \tan^{-1}\left(\frac{s_p}{f}\right) \cong \frac{s_p}{f} \tag{1.2}$$

where s_d and s_p correspond to photoreceptor spacing for the distal and proximal retinas and f is focal length (Land and Nilsson 2002). Rhabdoms were contiguous in the eyes of all species examined, letting $\Delta\varphi_d = \Delta\rho_d$ and $\Delta\varphi_p = \Delta\rho_p$, where $\Delta\rho_d$ and $\Delta\rho_p$ are the acceptance angles of the photoreceptors of the distal and proximal retina, respectively. The optical sensitivities of distal (S_d) and proximal (S_p) retinas were calculated using the formulas:

$$S_d = \left(\frac{\pi}{4}\right)^2 D^2(\Delta\rho_d)^2\, P_d(1 - P_d)(1 - P_p)^2 \quad \text{and} \tag{2.1}$$

$$S_p = \left(\frac{\pi}{4}\right)^2 D^2(\Delta\rho_p)^2\, P_p(1 - P_d)(1 - P_p) \tag{2.2}$$

where D is pupil diameter and the terms $(1 - P_d)(1 - P_p)^2$ and $(1 - P_d)(1 - P_p)$ account for the light that is absorbed as it passes through both retinas on the way to the mirror and through the proximal retina on the way back to the distal retina. This absorption of unfocused light effectively lowers sensitivity in the scallop eye. P_p and P_d are the fractions of light absorbed by the photoreceptors during one pass through the proximal and distal retinas, respectively. P_p and P_d were calculated using the formula:

$$P_{abs} = \frac{\int_{400}^{700} I(\lambda)(1 - e^{-kA(\lambda)l})d\lambda}{\int_{400}^{700} I(\lambda)d\lambda} \tag{3}$$

where $I(\lambda)$ is ambient irradiance (Kirschfeld 1974, Land 1981, Warrant and Nilsson 1998), k (=0.0067) is the absorption coefficient of the rhabdom, and l is rhabdom length (measured for distal or proximal photoreceptors where appropriate). For our calculations, we assumed that scallops live in environments dominated by green light, appropriate given estimated habitat depths in coastal waters (Table 1). We also assumed that scallops' eyes have peak sensitivity at 480 nm, based on evidence by Cronly-Dillon (1966).

Statistical analysis

Measurements of eye internal diameter, focal length (f), pupil diameter (D), photoreceptor spacing for the distal (s_d) and proximal (s_p) retinas, rhabdom length for the photoreceptors of the distal (l_d) and proximal (l_p) retinas, inter-receptor angle of the distal ($\Delta\varphi_d$) and proximal ($\Delta\varphi_p$) retinas, and optical sensitivity of the distal (S_d) and proximal (S_p) retinas were compared between *Placopecten magellenicus, Argopecten irradians*, and *Spondylus americanus* using Tukey-Kramer HSD multiple comparison tests (Zar 1999). Comparisons were not made between measurements for other scallop species due to insufficient sample sizes (Table 1).

RESULTS

Scallop eyes were located on the middle mantle fold at the distal ends of short tentacles. These eye-bearing tentacles lined the edges of the right and left valves from one end of the hinge to the other and were interspersed with longer, extensible sensory tentacles in all species. The eyes were surrounded by a pigmented epithelium, which was brown in *Amusium balloti*, blue in *Argopecten irradians*, and black in *Placopecten magellenicus, Chlamys hastata, Chlamys rubida, Crassadoma gigantea*, and *Spondylus americanus*. The corneas were composed of a monolayer of nucleated cells (Fig. 2). Corneal cells were cuboidal in all species except *C. gigantea*, in which they were columnar. Lenses were cellular in all species examined. The lenses of *A. balloti* and *P. magellenicus* were the largest observed and had front curvatures that were approximately hyperbolic, causing them to resemble those described for *Pecten maximus* (Linnaeus, 1758) by Land (1965). In contrast, the lenses of the other five species were relatively small and had front curvatures that were relatively spherical (Fig. 2). All scallop eyes contained the distinctive double retina described in detail in a number of past reports (Dakin 1910, Barber *et al.* 1966). Cells completely negative for alpha-tubulin staining were present in scallop retinas along with the photoreceptor cells. We suspect that these non-staining cells were glial cells (Barber *et al.* 1966), which generally serve to support neural cells and are

Table 1. Morphological measurements and calculations of optical sensitivity and inter-receptor angle, a measure of optical resolution, for the eyes of the swimming scallops *Amusium balloti*, *Placopecten magellenicus*, *Argopecten irradians*, *Chlamys hastata*, and *Chlamys rubida* and the sessile scallops *Crassadoma gigantea* and *Spondylus americanus*. Values represent mean ± 2 *SE*. Measurements and calculations for *P. magellenicus*, *A. irradians*, and *S. americanus* (appearing in bold columns) were compared statistically using Tukey-Kramer HSD multiple comparison tests. Significant differences between one species and the other two are denoted by * (if $\alpha = 0.05$) or ** (if $\alpha = 0.01$). Information regarding shell height, substrate type, habitat depth, and attachment type was adapted from Brand (2006), Lauzier and Bourne (2006), and personal observation (DIS). Shell height refers to the dorsal-ventral length of the valves.

	A. balloti ($n = 2$)	__*P. magellenicus*__ __($n = 16$)__	__*A. irradians*__ __($n = 16$)__	*C. hastata* ($n = 2$)	*C. rubida* ($n = 2$)	*C. gigantea* ($n = 3$)	__*S. americanus*__ __($n = 16$)__
Inter-receptor angle, distal retina $\Delta\varphi_d$ (°)	1.7 ± 0.1	__2.5 ± 0.2**__	__2.1 ± 0.1**__	2.5 ± 0.5	2.8 ± 0.1	3.0 ± 0.1	__3.6 ± 0.2**__
Inter-receptor angle, proximal retina $\Delta\varphi_p$ (°)	1.0 ± 0.1	__1.3 ± 0.1**__	__1.9 ± 0.2**__	2.5 ± 0.5	2.7 ± 0.3	3.2 ± 0.2	__4.5 ± 0.3**__
Optical sensitivity, distal retina S_d ($\mu m^2 \cdot sr$)	4 ± 1	__8 ± 1**__	__5 ± 1__	8 ± 5	6 ± 1	2 ± 1	__5 ± 1__
Optical sensitivity, proximal retina S_p ($\mu m^2 \cdot sr$)	3 ± 1	__4 ± 1**__	__11 ± 3**__	21 ± 10	10 ± 6	7 ± 3	__19 ± 4**__
Eye internal diameter (μm)	570 ± 30	__550 ± 40**__	__670 ± 40**__	480 ± 20	480 ± 20	450 ± 20	__370 ± 30**__
Pupil diameter D (μm)	390 ± 12	__350 ± 30*__	__400 ± 30*__	360 ± 40	310 ± 10	170 ± 20	__230 ± 20*__
Focal length f (μm)	170 ± 10	__150 ± 10**__	__180 ± 10**__	140 ± 20	110 ± 10	110 ± 10	__95 ± 6**__
Photoreceptor spacing, distal retina s_d (μm)	5	__6.4 ± 0.3__	__6.4 ± 0.2__	6	5.2 ± 0.4	6	__5.9 ± 0.3*__
Photoreceptor spacing, proximal retina s_p (μm)	3	__3.3 ± 0.2**__	__5.8 ± 0.2**__	6	5	6.3 ± 0.3	__7.4 ± 0.3**__
Rhabdom length, distal retina l_d (μm)	15 ± 2	__19 ± 3**__	__12 ± 2__	20	12 ± 1	14 ± 4	__13 ± 1__
Rhabdom length, proximal retina l_p (μm)	25	__33 ± 6__	__30 ± 10__	45	20 ± 6	40	__25 ± 2__
Shell height of specimens examined (cm)	?	__10__	__6__	6	5	10	__9__
Preferred substrate	sandy	__sandy__	__sandy__	rocky	rocky	rocky	__rocky__
Habitat depth (m)	10-75	__20-110__	__1-12__	2-150	1-200	1-80	__1-150__
Attachment type	unattached	__unattached__	__unattached__	byssal	byssal	cemented	__cemented__

not known to act in signal processing. The backs of all eyes were lined with a concave spherical mirror, again as described by Land (1965). Underlying the mirror was a red pigment layer. Contrary to past reports, we found that a cavity was present between the mirror and the retinas in all scallop species examined (Fig. 2). Cavity size varied greatly between species. Relatively small cavities were found in *A. balloti* and *P. magellenicus*, resulting in eyes that were morphologically similar to those of *P. maximus* (Land 1965), while larger cavities were present in the eyes of the other five species. Dissection and whole-mount microscopy revealed that the cavity was filled with a clear fluid.

Eye internal diameter, focal length (f), pupil diameter (D), photoreceptor spacing for distal (s_d) and proximal (s_p) retinas, and rhabdom length for the photoreceptors of the distal (l_d) and proximal (l_p) retinas varied between scallop species (Table 1). Swimming species generally had larger eyes, larger pupils, longer focal lengths, and proximal retina photoreceptors that were more closely spaced (Table 1). Rhabdom length and distal retina photoreceptor spacing did not appear to correlate with whether a species could swim or not (Table 1). Our calculations indicated that distal and proximal retina inter-receptor angle and optical sensitivity also differed between scallop species (Table 1). Swimming species tended to have smaller distal and proximal retina inter-receptor angles than sessile species (Table 1). Optical sensitivity did not appear to be related to scallop swimming ability.

DISCUSSION

Our study revealed several new aspects of scallop eye morphology. First, we found that lens size and shape varied between scallop species (Fig. 2). The lenses of *Amusium balloti* and *Placopecten magellenicus* had shapes similar to those

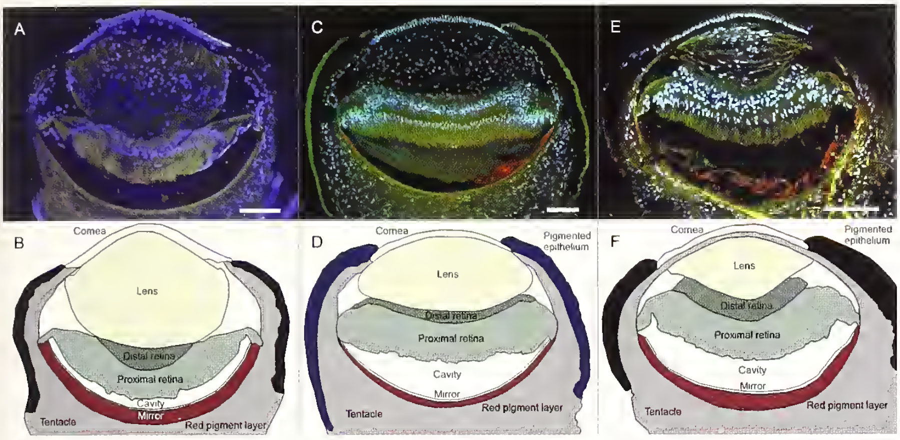

Figure 2. Mantle eye sections from the swimming scallops *Placopecten magellenicus* (A) and *Argopecten irradians* (C), imaged under a 10x confocal objective, and the sessile scallop *Spondylus americanus* (E), imaged under a 20x objective. Eyes were stained with Hoescht dye, causing cell nuclei to appear blue, and alpha-tubulin, causing microtubules to appear green. The pigment layer underneath the mirror appears red both in the images and *in vivo*. The diagrams (B, D, and F) correspond to the confocal images above and are labeled accordingly. The scale bars represent 100 μm.

described for *Pecten maximus* (Land 1965). The front of the *P. maximus* lens appears to be curved in such a way as to correct for spherical aberration caused by the reflection of light off the mirror (Land 1965), a function we will also attribute, tentatively, to the lenses of *A. balloti* and *P. magellenicus*. The lenses of the other five species appeared to have front curvatures that were relatively spherical, an indication that they may do little to correct for spherical aberration caused by the mirror. We are currently exploring the functional consequences of these different lens shapes and the phylogenetic distribution of lens types among a wide range of scallop species.

Second, we consistently noted a fluid-filled cavity between the proximal retina and the mirror in the eyes of all seven scallop species examined (Fig. 2). This cavity ranged in size between species. Small cavities were found in the eyes of *Amusium balloti* and *Placopecten magellenicus*, resulting in eyes that closely resembled those of *Pecten maximus* (Land 1965). Conversely, a large cavity was found between the proximal retina and the mirror in the eyes of the other five scallop species examined. The optics of the scallop eye are greatly influenced by the size of the cavity that exists between the proximal retina and the mirror. Following Land's analysis (1965) of the optics of *P. maximus*, which has eyes with a small cavity, it appears that focused light likely falls on the distal retina in the morphologically similar eyes of *A. balloti* and *P. magellenicus*. Alternately, due to the presence of a large cavity, it appears likely that focused light falls on the proximal retina in the eyes of the other scallop species we examined. We would be tempted to conclude that focused images simply fall on different retinas in different scallop species, but we have also found that photoreceptor spacing is tighter in the proximal retinas of *A. balloti* and *P. magellenicus* than it is in their distal retinas (Table 1). This is not consistent with a model in which the proximal retinas of *A. balloti* and *P. magellenicus* fail to receive focused light. We also found that *A. balloti* and *P. magellenicus* have the most tightly packed proximal retina photoreceptors of any of the species examined (Table 1), which again suggests that their proximal retinas may be involved in image formation. As an explanation for these inconsistencies, we speculate that scallop eyes are optically dynamic structures that can alternately focus light onto either of the two retinas through slight changes in shape. We are, at this time, exploring possible mechanistic bases for such a process.

An analysis of scallop visual capabilities provided evidence that swimming scallops have more acute vision than non-swimmers and that the best swimmers have the most acute vision. Among the scallops included in this study, *Amusium balloti* and *Placopecten magellenicus* were the strongest swimmers, capable of moving at speeds of up to 100 cm/s (Joll 1989) and 67 cm/s (Brand 2006), respectively. These scallops had proximal retina inter-receptor angles of around 1°, the smallest of any we calculated (Table 1). Weaker swimmers like *Argopecten irradians*, able to swim at speeds of 40 cm/s (Brand 2006), had proximal retina inter-receptor angles between 2-3° (Table 1). Our findings in this case concur with past morphological studies that found that *Pecten maximus*, a scallop with swimming abilities comparable to those of *A. irradians* (Brand 2006), had an optical resolution of about 2°. Sessile scallops, which cement to their substrate in a manner similar to oysters (Lauzier and Bourne 2006), had the largest proximal retina inter-receptor angles observed, at around 3-5° (Table 1). Proximal retina inter-receptor angle diversity was a product of differences in both focal length and photoreceptor spacing. For example, tighter photoreceptor packing was largely responsible for *A. balloti* having a smaller proximal retina inter-receptor angle than *A. irradians*, but longer focal length was responsible for *A. irradians* having a smaller proximal retina inter-receptor angle than *Chlamys hastata*. Factors other than swimming ability may also help explain why some scallop species have better optical resolution than others. For example, scallops from sandy substrates tend to have better vision and be better swimmers than those from rocky habitats (Table 1). Another important caveat is that our methods have led us to estimate the theoretical maximum of visual acuity in each scallop species. Neural processes, like spatial summation, and optical imperfections, such spherical aberration, may lead to scallops having actual visual acuities that are below these estimates (Land and Nilsson 2002). However, behavioral (Buddenbrock and Moller-Racke 1953) and electrophysiological (Land 1966) studies on *P. maximus* provide evidence that actual scallop visual acuity is close to the theoretical maximum derived from focal length and photoreceptor spacing. This suggests that our estimates of inter-receptor angle likely point towards true functional differences between the eyes of mobile and immobile scallop species. Finally, interspecific differences in inter-receptor angle will have little consequence if focused light falls on different retinas in different scallop species, a possibility that we address in detail above.

Distal retina inter-receptor angles, ranging from 1.7 ± 0.1° for *Amusium balloti* to 3.6 ± 0.2° for *Spondylus americanus*, only varied two-fold between species, as opposed to the four-fold difference observed between proximal retina inter-receptor angles (Table 1). Distal retina inter-receptor angle also correlated with scallop swimming ability but not as strongly as proximal retina inter-receptor angle did. For example, proximal retina inter-receptor angle was larger in *Placopecten magellenicus* than it was in *Argopecten irradians*, despite *P. magellenicus* being the stronger swimmer (Brand 2006). Perhaps more tellingly, variation in distal retina inter-receptor angle was largely a product of interspecific differences in focal length, not photoreceptor spacing. Distal retina photoreceptor spacing fell between 5 and 6.5 μm in all species and, unlike proximal retina photoreceptor spacing, a relationship between this measure and a scallop species' swimming ability was not indicated by the data (Table 1).

It has been suggested that the two scallop retinas perform different visual functions (Land 1966, Wilkens 2006), in part due to evidence that the retinas operate via different opsins and signal-transduction pathways (Kojima *et al.* 1997) and that the neurons of the distal retina hyperpolarize in response to light, while those of the proximal retina depolarize (Hartline 1938, Land 1966, McReynolds and Gorman 1970). This proposal is supported by our evidence that proximal retina photoreceptor spacing may depend on a scallop species' swimming ability, while distal retina photoreceptor spacing varies little between species (Table 1). This implies that scallop proximal retinas may be involved in visual tasks more important to swimming species, such as those relating to the detection of preferred habitat, and that the distal retinas are likely involved in tasks of equal importance to both swimming and sessile species, such as predator detection.

Further support for functional differentiation of this sort comes from indications that scallop proximal retinas are better at gathering information about relatively static environmental features (Land 1966), like the eelgrass beds towards which *Argopecten irradians* has been found to swim (Hamilton and Koch 1996), while the distal retinas are better at detecting movement, such as that by potential predators.

Unrecognized differences between the eyes of mobile and immobile species have contributed to arguments that swimming scallops do not visually detect preferred habitats, as has the fact that scallops lack a centralized nervous system (Morton 2000). While it is true that scallops do not process much visual information in their brain, their visceroparietal ganglion (VPG) contains optic lobes that likely give these animals the neural capacity to convert a range of visual inputs into behavioral output (Wilkens 2006). It has been noted that information from the proximal retina elicits greater activity in the VPG's optic lobes than information from the distal retina (Wilkens and Ache 1977), a finding seemingly at odds with the claim that focused light only falls on the scallop distal retina (Land 1965). As a potential solution to this problem, we suggest that focused light may fall on the proximal retina in at least some scallop species. This suggests that previously unrecognized interspecific variation may account for inconsistencies between past reports. It also suggests that the scallop optic lobes may, at least in some cases, process visual information from the proximal retina for the sake of complex behavioral tasks like habitat detection.

Scallop optical sensitivity, like optical resolution, differed between retinas and between species (Table 1). However, unlike optical resolution, optical sensitivity did not appear to correlate with swimming ability or, as might be expected, with habitat depth (Table 1). Given that irradiance values in scallop habitats may vary over several orders of magnitude, depending on tide conditions and time of day, the differences we observed between optical sensitivities may have only minor functional consequences for the species examined in this study.

In conclusion, we found that eye morphology varied between scallop species and that swimming scallops tend to have better vision than sessile scallops. This latter discovery is consistent with our hypothesis that mobile scallops may visually detect preferred habitats. We also found evidence that scallop distal and proximal retinas may be functionally differentiated. We are currently working to clarify the relationship between vision and swimming ability in scallops and to develop new models of scallop optics that focus, in particular, on the range of lens shapes we have observed in different scallop species and on the ways that scallops may utilize both of their retinas for image formation.

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The evolution of eyes in the Bivalvia: New insights*

Brian Morton

Department of Zoology, The Natural History Museum, Cromwell Road, London SW7 5BD, U.K., prof_bmorton@hotmail.co.uk

Abstract: Two types of multi-cellular eyes have been identified in the Bivalvia. Paired cephalic eyes occurring internally above the anterior end of the ctenidia are seen only in representatives of the Arcoidea, Limopsoidea, Mytiloidea, Anomioidea, Ostreoidea, and Limoidea. These eyes, comprising a pit of photo-sensory cells and a simple lens, are thought to represent the earliest method of photoreception. Many shallow-water marine, estuarine, and freshwater bivalves also possess simple photoreceptive cells in the mantle that enable them to respond to shadows. In some other marine, shallow-water taxa, however, a second type of more complex photoreceptors has evolved. These comprise ectopic pallial eyes that can be divided into three broad categories, in terms of their locations on the (i) outer mantle fold in representatives of the Arcoidea, Limopsoidea, Pterioidea, and Anomioidea, (ii) middle fold in the Pectinoidea and Limoidea, and (iii) inner fold in the Cardioidea, Tridacnoidea, and Laternulidae (Anomalodesmata). Eyes do not occur in deep-sea bivalve taxa. Where ectopic pallial eyes occur, they measure amounts of light and integrate intensities from different directions, thereby supplying information to the individual possessing them about the distribution of light in its immediate environment. This does not mean, however, despite broad, phylogenetically related advances in pallial eye complexity, that any bivalve can perceive an image. A revised picture of the independent evolution of ectopic pallial eyes in the Bivalvia is provided. In bivalves, pallial fold duplication has resulted in improvements to the peripheral visual senses, albeit at different times in different phylogenies and on different components of the mantle margin. This has been achieved, it is herein argued, through: (i) selective gene-induced ectopism; (ii) pigment cup evagination in Category 1 eyes; (iii) invagination in Categories 2 and 3; and (iv) natural selection. The invaginated distal retina in representatives of the Pectinidae and Laternulidae provides the potential for image formation and the detection of movement. In the absence of optic lobes capable of synthesizing such information, however, these complex eyes must await matching cerebral sophistication.

Key words: cephalic and pallial eyes, evagination, invagination, duplication, mantle folds

A wide range of internal proprioreceptors and external sense organs has evolved in the Bivalvia. The former includes muscle proprioreceptors to moderate muscle tonus and, uniquely, in the watering pot shells (Clavagellidae and Penicillidae), a pair of pericardial proprioreceptors to monitor body tonus (Morton 2007). The latter sense organs include paired statocysts (for orientation), osphradia for the reception of chemical spawning cues and synchronization of gamete emission (Beninger *et al.* 1995), abdominal (Haszprunar 1985) and pallial sense organs, as in *Aulacomya atra* (Molina, 1782) (Zaixso 2003), both for the mechanoreception of water currents, and light-sensitive cephalic and pallial eyes (Morton 2001). These are structures that can measure amounts of light and integrate intensities from different directions, thereby supplying information to the individual possessing them about the distribution of light in its immediate environment (Land and Nilsson 2002). As will be argued, however, the presence of such light-sensitive structures does not necessarily mean, despite phylogenetically related advances in pallial eye complexity, that any bivalve eye, in the absence of significant integrative optic lobes in the cerebral ganglia, can perceive an image of its immediate environment.

When present, cephalic eyes are located internally on the left and right sides of the body at the base of the anterior-most filaments of the inner demibranchs of the ctenidia but have been seen and described only for representatives of the Arcoidea, Limopsoidea, Mytiloidea, Anomioidea, Ostreoidea, and Limoidea (Morton 2001). The eyes, each comprising a pit of photo-sensory cells and a simple lens, are thought to represent the earliest method of photoreception in the Bivalvia, but their functional efficiency must be constrained by the fact that, in life, light would be received by them after transmission through the thickest part of the shell in epibenthic taxa and from the posterior in the case of most infaunal species. More recent bivalves do not possess such eyes (Morton 2001).

Kennedy (1960) described the simplest pallial photoreceptor known for the Bivalvia. He demonstrated that the pallial nerves of *Spisula solidissima* (Dillwyn, 1817) each contain a single light-sensitive afferent nerve fiber. This responds directly to illumination and mediates the shadow response (or reflex) of siphonal retraction. Because the shadow reflex is so widespread in freshwater and coastal bivalves, it can be assumed that most of them possess such sensory fibers. Even, for example, in the absence of any obvious photoreceptors, *Donax vittatus* (da Costa, 1778) responds to changes in incident light intensity by adjusting its position in the sediment (Ansell *et al.* 1998). Conversely, the subterranean freshwater dreissenid *Congeria kusceri* Bole, 1962 does not possess such a reflex (pers. obs.) and no multi-cellular pallial eyes have evolved in freshwater, brackish-water, or deep-water bivalves. Only intertidal and shallow continental shelf species possess them.

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A few other coastal bivalves have, however, evolved more sophisticated pallial photoreceptors. In a few taxa, such structures have resulted in the evolution of pallial eyes of extraordinary (for such sedentary creatures) structural complexity. Morton (2001) reviewed such eyes in the Bivalvia and showed that pallial photoreceptors could be divided into three categories (typically associated with an increasing degree of morphological specialization) based upon their locations on the (i) outer, (ii) middle, or (iii) inner mantle folds. As will be also discussed, some progress has been made in understanding how the development of eyes is controlled genetically, most recently reviewed by Gehring (2001), but the objectives of this paper are to understand (i) how the pattern of increasing pallial eye sophistication has come about and (ii) how, for each category of eye (Morton 2001), its various components have been assembled.

CEPHALIC EYES

It is believed that molluscan cephalic eyes can be derived from structures similar to those that occur in planarians, themselves representative of those that would have been found in the ancestor(s) of all metazoans. Gehring (2001), quoting Hesse (1897), believes that the eyes of *Planaria torva* are the most likely candidates for and examples of such an ancestor. Each *P. torva* eye comprises three photoreceptor cells surrounding a single pigment cell (Fig. 1). The cephalic eyes of bivalves are only somewhat more complex and the larvae of oysters, for example, *Crassostrea virginica* (Gmelin, 1791) and *Crassostrea gigas* (Thünberg, 1793), develop a pair of light-sensitive eyespots (Nelson 1926) that gradually start to degenerate soon after metamorphosis (Baker and Mann 1994). In species of *Chlamys* Röding, 1798, *Pecten* Müller, 1776, and *Ostrea* Linnaeus, 1758, such eyespots consist of a cup of pigmented cells surrounding an amorphous (unstructured) lens (Cole 1938, Cragg and Crisp 1991, Hodgson and Burke 1998). Cole (1938) has described the cephalic eye in the pre-settlement veliger of *Ostrea edulis* Linnaeus, 1758 (Fig. 2) and notes that each one is situated on the body wall just dorsal to where the gill rudiments attach and, oppositely, to the mantle.

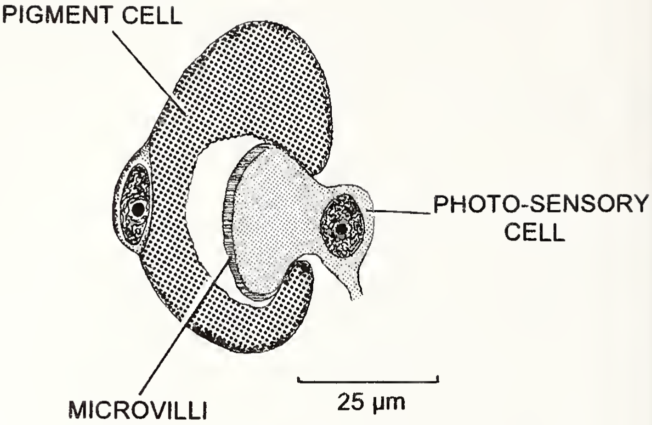

Figure 1. A section through the eye of the flatworm *Planaria torva*. Redrawn after Hesse (1897).

Cephalic eye structure in the Bivalvia appears to be highly uniform (Morton 2001). This suggests that such eyes must have a common ancestry and may once have been much more widespread in the class but have subsequently either been lost or never developed in the majority of taxa. Those of *Aulacomya atra* (Mytilidae) can serve as an example of cephalic eye structure and have been described by Zaixso

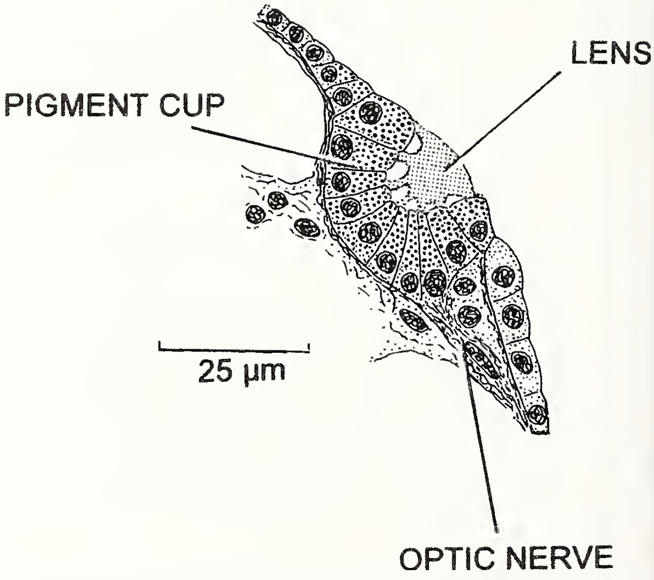

Figure 2. A section through the cephalic eye of the pre-settlement veliger of *Ostrea edulis*. Redrawn after Cole (1938).

(2003). One such eye is illustrated in section (Fig. 3). It comprises a cup of pigmented and photo-sensory cells enclosing a simple, amorphous, lens. Nerve fibers arise basally from the photo-sensory cells to connect up with the cerebropleural ganglia. Such an eye structure is similar to that of *Philobrya munita* (Finlay, 1930) and of *Pteria brevialata* (Dunker, 1872) (Morton 1978, 1995).

PALLIAL EYES

Category 1: Pterioidea, Arcoidea, Limopsoidea, and Anomioidea

Pterioidea: Pteria brevialata

Morton (1995) has described the mantle margin and pallial eyes of *Pteria brevialata* (Pteriidae). Although the mantle margin appears to comprise the usual bivalve three folds (Yonge 1982), it does not. Instead, the outer mantle fold is sub-divided into two sub-folds comprising (i) a specialized inner photo-sensory component on which are located the pallial eyes (Category 1: Morton 2001) and (ii) external to this an outer component that secretes the shell. Both sub-folds are, therefore, overlain by the shell and two-layered periostracum when the mantle is retracted and the pallial eyes thus monitor light even through the periostracum when the mantle margin is extended. The pallial eyes of *P. brevialata* are among the simplest multi-cellular optical structures yet described for the Bivalvia and comprise simple photo-sensory cells located on the outer epithelium of the inner component of the outer mantle fold and are backed on the opposite, inner, epithelium by a patch of pigmented cells (Fig. 4). Nerve fibers arise from the bases of the photo-sensory cells and pass towards the pallial nerve.

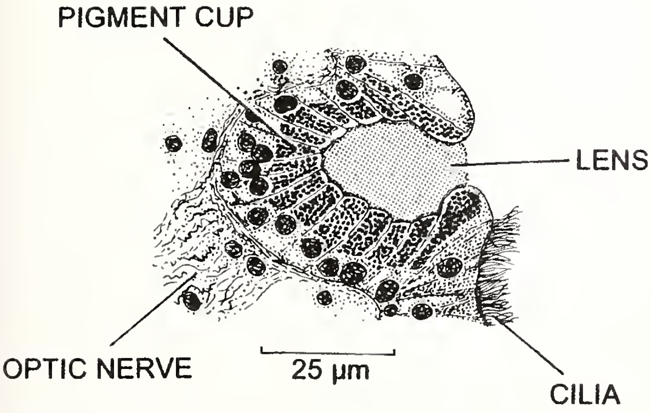

Figure 3. A section through a cephalic eye of *Aulacomya atra*. Redrawn after Zaixso (2003).

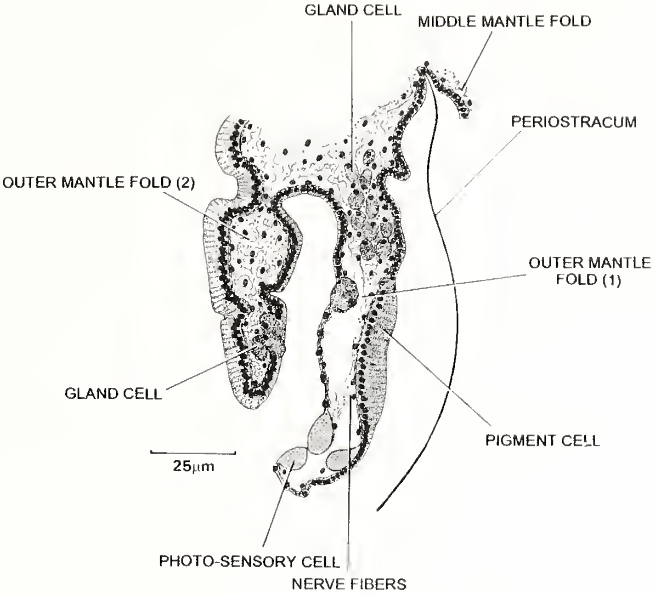

Figure 4. A section through the simplest known pallial eye of the outer mantle fold of *Pteria brevialata* (Category 1). Redrawn after Morton (1995).

Arcoidea and Limopsoidea

Representatives of the Arcoidea (and Limopsoidea) possess two types of pallial eyes, namely multi-faceted compound eyes and simple pigment cups. Both types may occur in the same species but both develop on the inner sub-fold of the outer mantle fold and are, as a consequence, covered by the periostracum even when the mantle margins are expanded, as in *Pteria brevialata* described above.

Arca noae

The mantle margin of *Arca noae* Linnaeus, 1758 is illustrated in section (Figs. 5 and 6B) (after Morton and Peharda 2008). The pallial eyes of *A. noae* are of the compound ommatidial type first described by Waller (1980, 1981), their fine structure subsequently elucidated by Nilsson (1994). Each, of many hundreds, compound eye of *A. noae* is located on the inner sub-fold of the outer mantle fold and comprises photosensitive cells surrounded by and intermixed with pigment cells. In *A. noae*, the plump stalk and mantle beneath each eye are also pigmented, perhaps enhancing their photosensory efficiency.

Barbatia virescens

The second eye type seen in representatives of the Arcidae (and Limopsoidea) is a pigment cup or invaginate eye. One of the pallial eyes of *Barbatia virescens* (Reeve, 1844) (Fig. 6A), also located on the inner sub-fold of the outer mantle fold (Morton 1987), comprises a simple cup of intermixed photo-sensory cells and pigment cells. The cup

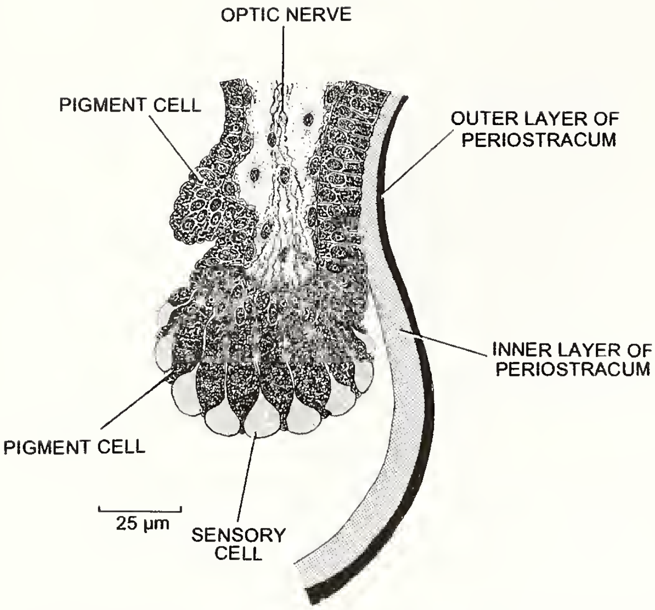
OPTIC NERVE
PIGMENT CELL
OUTER LAYER OF PERIOSTRACUM
INNER LAYER OF PERIOSTRACUM
PIGMENT CELL
25 µm
SENSORY CELL

Figure 5. A section through the pallial eye of the outer mantle fold of *Arca noae* (Category 1). Redrawn after Morton and Peharda (2008).

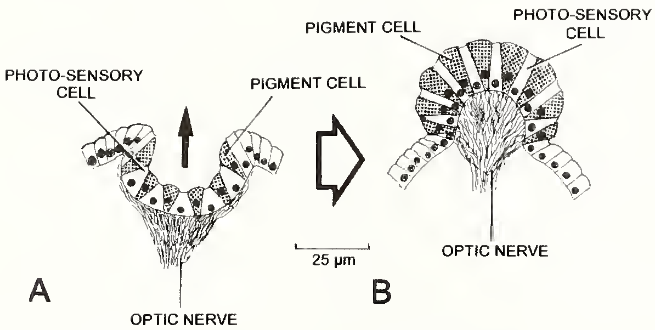
PHOTO-SENSORY CELL
PIGMENT CELL
PIGMENT CELL
PHOTO-SENSORY CELL
25 µm
OPTIC NERVE
A
OPTIC NERVE
B

Figure 6. Sections through the simple cup-like pallial eye of A, *Barbatia virescens* (Redrawn after Morton 1987) and B, the ommatidial pallial eye typical of representatives of the Arcoidea (both Category 1).

encloses an amorphous lens and the eye is thus similar to the bivalve cephalic eye.

As will be discussed, the compound ommatidial eye (Fig. 6B) might have evolved by evagination from the simpler invaginate eye (Fig. 6A). For example, the limopsid *Philobrya munita* (Finlay, 1930) has a pallial eye that is possibly intermediate between the inverted cup and compound ommatidial eyes (Morton 1978, fig. 4).

Anomioidea: Enigmonia aenigmatica

The pallial eyes of *Enigmonia aenigmatica* (Holten, 1803) (Anomiidae) are located, uniquely, on the general mantle beneath the shell (Morton 1976, fig. 2) and, thus, also the periostracum and have been described by Morton (2001, figs. 8 and 9). Each adult individual possesses ~22 cups of pigmented retinal cells that have invaginated from the inner epithelium of the general mantle surface. Above each cup is a more structured, cellular lens that is formed on the outer surface of the general mantle. There is also a ciliated accessory sense organ. It is unknown how this optical structure has been assembled from either (i) an invaginated cephalic eye pigment cup that has moved from its usual position or (ii) has migrated inwards from the mantle margin. The occurrence with each eye of an accessory ciliated sense organ is, however, unusual in that, as will be seen, such a structure is typical only of Category 3 eyes (those that occur on the inner mantle fold). Bearing in mind that *E. aenigmatica* lies on its right shell valve, it seems possible that the eyes have, in evolutionary terms, migrated inwards from the left mantle lobe to lie under the left shell valve. From which marginal fold, however, is unknown. The fact that the eyes perceive light through the shell, however, places them in Category 1 but they do constitute a unique photosensory structure in the Bivalvia.

Category 2: Limidae and Pectinidae

Limidae: Ctenoides floridanus

The pallial eyes of *Ctenoides floridanus* (Olsson and Harbison, 1953) are located on the middle mantle fold and have been described by Morton (2000a). One is illustrated in section (Fig. 7A). Each eye comprises a cup of photo-sensory cells surrounded laterally by pigment cells. Each sensory cell of *Lima scabra* (Born, 1778) has been shown by Bell and Mpitsos (1968) to possess bundles of cilia and basal neural processes. A large optical nerve arises from the base of the cup and there is a cellular lens. Interestingly, however, the

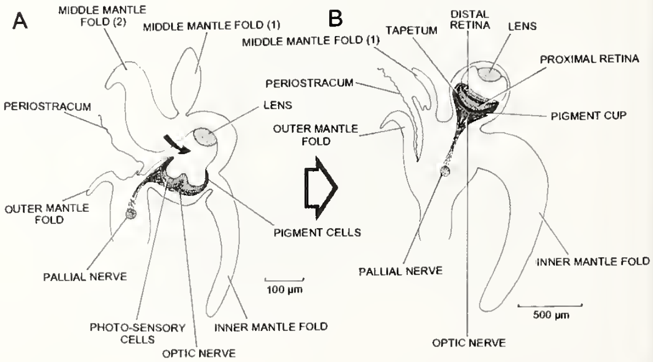
A
MIDDLE MANTLE FOLD (2)
MIDDLE MANTLE FOLD (1)
PERIOSTRACUM
LENS
OUTER MANTLE FOLD
PIGMENT CELLS
OUTER MANTLE FOLD
PALLIAL NERVE
100 µm
PHOTO-SENSORY CELLS
INNER MANTLE FOLD
OPTIC NERVE
B
MIDDLE MANTLE FOLD (1)
TAPETUM
DISTAL RETINA
LENS
PROXIMAL RETINA
PERIOSTRACUM
PIGMENT CUP
PALLIAL NERVE
INNER MANTLE FOLD
500 µm
OPTIC NERVE

Figure 7. Simplified sections through the pallial eyes (Category 2) of A, *Ctenoides floridanus* and B, *Pecten pusio*. Redrawn after Morton (2001) and Patten (1886), respectively.

eye is not closed to the sea, but is open, and an amorphous plug of tissue occupies the space between retina and lens.

The importance of this invaginated eye is that it illustrates, as will be discussed, the manner in which bivalve ectopic pallial eyes have changed their locations in the Pteriomorphia from a specialized sub-fold of the outer mantle fold (and thus beneath the shell and periostracum) to the middle fold (and thus beyond the shell and periostracum).

Pectinidae: Pecten pusio

The eye of *Pecten pusio* (Linnaeus, 1758) (Fig. 7B) has been described by Patten (1886), and Dakin (1910, 1928) and Morton (1980, 1993, 1996, 2000b) have described the eyes of other pectinids, including species of *Spondylus* (Linnaeus, 1758), *Amusium* Röding, 1798, *Leptopecten* Verrill, 1897, *Minnivola* Iredale, 1939, and *Placopecten* Verrill, 1897. The eyes are located on the middle mantle fold with more on the mantle margin of the upper right valve than the lower left [see, for example, the description of *Amusium pleuronectes* (Linnaeus, 1758) by Morton (1980)].

The pectinid eye is the most well-known bivalve optical structure and has best been described for *Pecten maximus* (Linnaeus, 1758) in a series of papers by M. F. Land (see Morton 2001 for a review). This species and *Pecten pusio* uses a lens to focus light onto a parabolic mirror of pigmented cells, called the argentea, and reflecting this back onto the more distal of two, ciliary-based, retinal layers (Land 1965) where it is capable of defining an image. The proximal retina, on the other hand, detects changes in light intensity (Land 1966). Each retina possesses cilia with a 9 + 0 structure but the proximal one will be responsible for stimulating the typical escape response of free-living species of the Pectinidae, for example, the accomplished swimmer *Amusium pleuronectes* (Morton 1980).

Category 3: Myidae, Cardiidae, Tridacnidae, and Laternulidae

Myidae: Mya arenaria

Light (1930) demonstrated for *Mya arenaria* Linnaeus, 1758 that photosensitive tissue is located in the inner surface of the siphons, mostly at their tips, that is, the fused inner mantle folds. He further identified using silver nitrate staining and described light sensitive cells each containing an optic organelle composed of a large hyaline structure—the lens—surrounded by a network of nerve fibers that he called the retinella. Two of these cells are illustrated (Fig. 8).

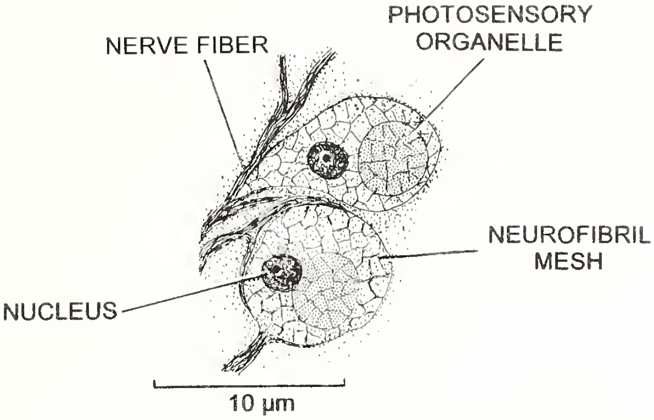

Figure 8. *Mya arenaria.* A section through two siphonal photoreceptors. Redrawn after Light (1930).

Cardiidae and Tridacnidae: Cerastoderma edule *and* Tridacna maxima

The pallial eyes of representatives of the Cardiidae have been described by numerous authors, but the eyes of *Cerastoderma edule* (Linnaeus, 1758) are the most well-known (Barber and Land 1967, Barber and Wright 1968). In an adult individual, ~60 of them occur on the inner folds of both mantle lobes each atop a siphonal tentacle. Each eye comprises a cup of pigmented cells enclosing 12-20 receptor cells. Each photo-sensory receptor cell has about 100 cilia, each with a 9 + 0 filament content (Barber and Land 1967) and with the optical nerve arising internally and departing the eye at the apical junction of the cup with the lens. Joining the optic nerve laterally is a nerve from a ciliated sense organ of unknown function. It is probably, however, a mechanoreceptor. Weber (1909) and Braun (1954, fig. 5) showed that the accessory sense organ of each pallial eye of *Cardium oblongum* Gmelin, 1791 actually comprises two clusters of cilia (Fig. 9). This is also true for the pallial eye of *C. edule* (Fig. 10A).

Representatives of the Tridacnidae also possess pallial eyes that are located as in the Cardiidae on the, albeit greatly expanded, inner mantle folds. An adult *Tridacna maxima* (Röding, 1798) will have thousands of eyespots but they have the same basic structure as those described (above) for representatives of the Cardiidae. As well as fulfilling a visual function, however, tridacnid eyes may focus light on masses of zooxanthellae residing around them in the mantle hemocoel, prompting Yonge (1936) to refer to them as hyaline organs (although still derived from true eyes). The light-receiving properties of the array of tridacnid eyes has been demonstrated by Stasek (1965) who showed that the shadow reflex results in a giant clam squirting a jet of 'aimed' water at any fish (or human) moving above it. The zooxanthellae might also, however, function as an accessory light reflecting pigment cup, enhancing the efficiency of the clearly photosensory eye apparatus and thereby confirming the truly symbiotic relationship that exists between the bivalve and its entrained phytoplankters.

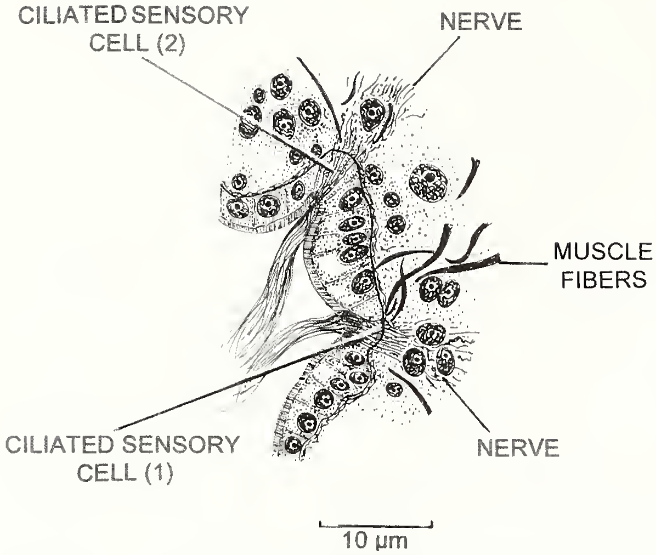

Figure 9. A section through the accessory, ciliated, sense organ of the pallial eye (Category 3) of *Cardium oblongum* Gmelin, 1791. Redrawn after Braun (1954).

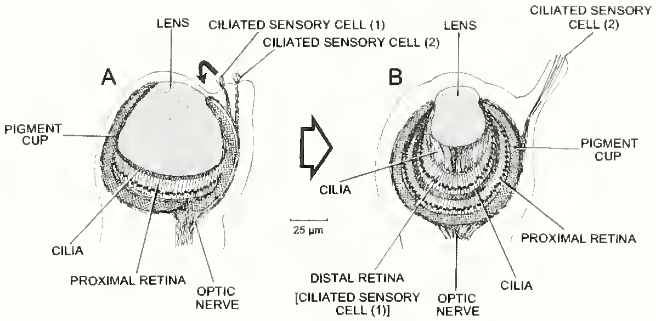
LENS
CILIATED SENSORY CELL (1)
CILIATED SENSORY CELL (2)
LENS
CILIATED SENSORY CELL (2)
A
B
PIGMENT CUP
PIGMENT CUP
CILIA
25 µm
CILIA
PROXIMAL RETINA
PROXIMAL RETINA
OPTIC NERVE
DISTAL RETINA [CILIATED SENSORY CELL (1)]
OPTIC NERVE
CILIA

Figure 10. Simplified sections through the pallial eyes (Category 3) of *Cerastoderma edule* (after a number of authors) and *Laternula truncata*. Redrawn after Adal and Morton (1973).

Laternulidae: Laternula truncata

The Anomalodesmata comprises the most family-rich subclass of the Bivalvia and yet only representatives of the Laternulidae, for example *Laternula truncata* (Lamarck, 1818), have been shown to possess pallial eyes (Adal and Morton 1973) (Fig. 10B). As in *Cerastoderma edule*, each of the nine eyes sits atop a siphonal tentacle and is hence located on the inner mantle fold. As in representatives of the Pectinidae, for example *Pecten maximus* (Dakin 1910), there is a cup of pigment cells forming a parabolic mirror, or argentea, which encloses a double-layered, proximal and distal retina. The lens focuses light onto the argentea, which reflects it back onto the distal retina. In such a way, it is possible, as in representatives of the Pectinidae, to form an image. Morton (1973) showed, however, that the extremely sedentary infaunal *L. truncata*, which is capable of only slow burrowing, possesses only a shadow reflex that results in pallial tentacles attempting to flick sand grains over the exposed siphons. Presumably, if the situation is similar to that seen in representatives of the Pectinidae, the proximal retina is responsible for detecting changes in light intensities and thus stimulating the shadow reflex. The cells of each retina possess cilia that, again as in representatives of the Pectinidae, have a 9 + 0 structure (Adal and Morton 1973).

As in *Cerastoderma edule*, each pallial eye of *Laternula truncata* has an accessory sense organ except that in this species the groups of ~28 cilia with a 9 × 0 + 2 structure are long (50 µm) and contained within an invagination of a specialized tentacle (Adal and Morton 1973). Projecting from a pore at the apex of the tentacle, each cilium may make contact with the microvilli of the surrounding epithelium (as the tentacle moves) and is, hence, probably a mechano-receptor. The eyes of *L. truncata* represent a remarkable example of convergent evolution with those of representatives of the Pectinidae.

DISCUSSION

Gaten (1998) suggested that superimposition eyes, which re-direct light from many facets onto the target rhabdomeres, have evolved only once in the crustacean Decapoda, probably in the Devonian (345-395 mya). Morton (2001) similarly argued that in the Bivalvia, cup-like, cephalic eyes have probably also only evolved once but have been either retained by or developed in representatives of only a few (generally older) phylogenies (see above). Morton (2001) also showed, however, that, conversely, ectopic pallial eyes have evolved a number of times in various lineages of the Bivalvia and, typically, in different ways, notably with regard to their positions on the various folds of the mantle margin.

The sizes (diameters) of various bivalve pallial eyes are illustrated (Fig. 11) in relation to their category. What is obvious is that there is no clear relationship between eye category and size. That is, Category 1 eyes are all <~800 µm in diameter whereas Category 2 eyes all range in diameter between 100 µm and <1,000 µm. Category 3 eyes, however, are also only ~100 µm in diameter. Size does not seem to be an indicator of structural and, hence, optical complexity even though the pallial eyes of *Pecten maximus* (a large scallop) are among the largest and most sophisticated bivalve optical structures. Moreover, the pallial eyes of *Laternula truncata* are only 100 µm in diameter but are as morphologically complex as those of *P. maximus* (Adal and Morton 1980).

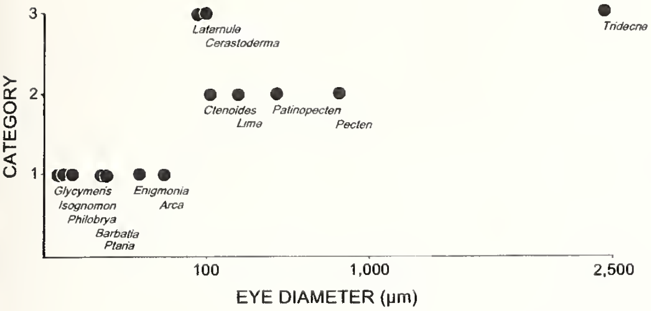

Figure 11. The pallial eye diameters of bivalves which are known to possess them and which have been divided into the three structural categories of Morton (2001). Note: x-axis scale is not uniform.

Furthermore, the eyes of representatives of the Tridacnidae (Fig. 11) are ~2.5 mm in diameter and hence about 25 times the size of those of their nearest relatives, the cardiid cockles. Although giant clams are the largest extant bivalves, their pallial eyes also have the same basic structure as representatives of the Cardiidae. It is, therefore, possible that tridacnid eyes are larger because, as well as fulfilling a visual function, they focus light on masses of zooxanthellae residing around them in the mantle hemocoel (Fankboner 1981, Wilkens 1986). The zooxanthellae might also, however, function as an accessory light-reflecting pigment cup. Excluding, therefore, as a special case, the hyaline organs/eyes of the Tridacnidae, it seems (Fig. 11) that all bivalve ectopic pallial eyes are of about the same size, probably in relation to the size of the individuals that possess them. Thompson (1942: 34-35) noted for a number of vertebrates that:

> *"A big dog's eye is hardly bigger than a little dog's; a squirrel's is much bigger, proportionately, than an elephant's; a robin's is but little less than a pigeon's or a crow's."*

Hence, as with Thompson's vertebrate examples, size alone is not an indication in the Bivalvia of either structural complexity or visual acuity. Nevertheless, the fact that some bivalves, for example representatives of the Arcoidea, Pectinidae, and Tridacnidae, possess so many pallial eyes suggests that they are highly important in another way. First, however, what controls pallial eye development?

The *Six1/2/so* and *Six3/6/7/9/optix* groups of genes are implicated in eye and sensory structure development. In *Drosophila*, *so* is associated with the eye marginal disc and Bolwig's organ, and the expression of *so* in the un-patterned epithelium is required for eye morphogenesis and development of the visual system (Cheyette *et al.* 1994). A mutation in the *so* homeobox disrupts the larval visual system of *Drosophila* and abolishes the larva's response to light (Hassan *et al.* 2000). The expression of *so* is regulated by *eyes absent* (*eya*) (Bonini *et al.* 1997) and the expression of both is required to induce ectopic eyes (Halder *et al.* 1998). However, Gehring and Ikeo (1999) considered *Pax6* and its homologs to be the control gene for eye development in metazoans although it is now known that a number of others (including *eya*), for example, *dachshund* (Shen and Mardon 1997) and *sine oculis* (Pignoni *et al.* 1997) are also able to induce ectopic eye expression in *Drosophila*. Serb (2008, fig. 1) provides a model of the network of genes that regulates eye formation in *Drosophila*.

In the case of the Bivalvia, however, the recovery of *so* sequences from *Nutricola tantilla* (Gould, 1853) and *Crassostrea gigas* by Bebenek *et al.* (2004) suggests that the ability to develop ectopic eyes is present in both taxa but neither has produced them, possibly because either *eya* or the other genes identified above are not present. Such a generalization might apply to the vast majority of bivalves (Fig. 12). However, Light (1930) appears to be the only person who has, once determining that *Mya arenaria* does have a shadow reflex, demonstrated, using silver nitrate staining, the presence and described the structure of intra-cellular light sensitive bodies mostly in the tips of the siphons. Since it is known that most shallow-water marine, estuarine, and freshwater bivalves do possess a shadow reflex, perhaps it would be beneficial if they too could be examined in more detail prior to arguing that *eya* and/or the other genes now associated with ectopic eye development (in *Drosophila*) are not present.

As described by Nilsson (1994), for representatives of the Arcidae, and reviewed by Morton (2001), the pallial eyes of those bivalves that possess them, regardless of their structural complexity, are analogous to photoreceptive burglar alarms that are placed on the outsides of (human) buildings. That is, although no image is discerned, only simple spatial movement, the efficiency of this optical sensory system has been refined in each of the three categories of eyes. How?

In the Arcoida, pallial eyes are restricted to species that inhabit shallow waters so that the deep-water *Bathyarca pectunculoides* (Scacchi, 1833) does not possess them (Morton 1982). The pallial eyes of *Barbatia virescens* and *Anadara notabilis* (Röding, 1798) are simple ciliated pits (Morton 1987, Nilsson 1994) whereas the compound ommatidial eyes of *Arca noae* conform in structure to those of other species of *Arca* (Waller 1980, 1981) and *Barbatia cancellaria* (Lamarck, 1819) (Nilsson 1994). Since the pallial eyes of representatives of the Arcoida (Category 1) comprise pigment cups which lack a well-defined lens and the cavity is filled with rhabdomeric microvilli arising from the receptor cells (Nilsson 1994), there are no cilia so that there is no base structure present for the evolution of a ciliary-based retina. Notwithstanding, an arcid compound ommatidial eye can be derived by evagination of a pigment cup (as illustrated in

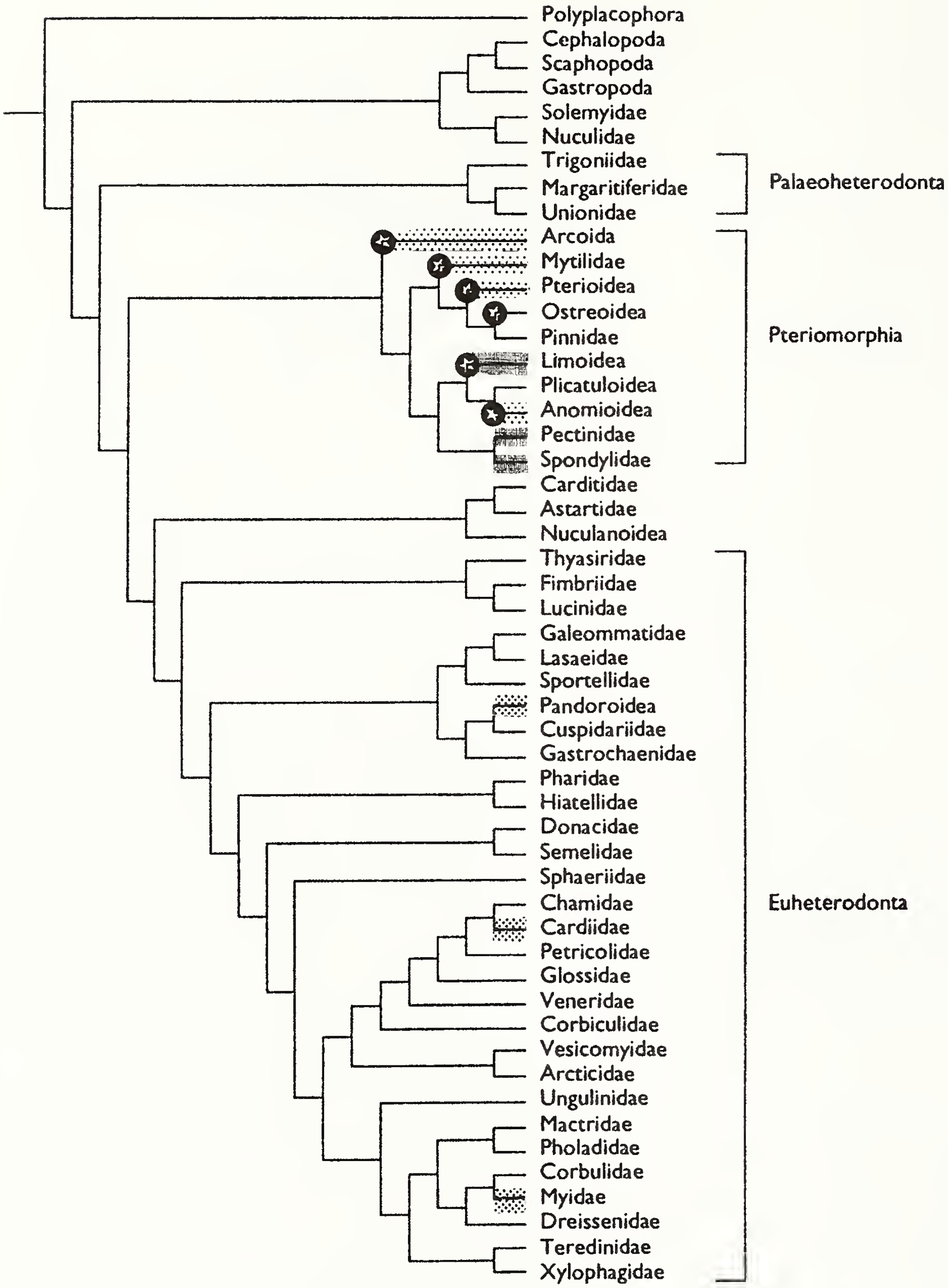
Polyplacophora
Cephalopoda
Scaphopoda
Gastropoda
Solemyidae
Nuculidae
Trigoniidae
Margaritiferidae
Unionidae
Arcoida
Mytilidae
Pterioidea
Ostreoidea
Pinnidae
Limoidea
Plicatuloidea
Anomioidea
Pectinidae
Spondylidae
Carditidae
Astartidae
Nuculanoidea
Thyasiridae
Fimbriidae
Lucinidae
Galeommatidae
Lasaeidae
Sportellidae
Pandoroidea
Cuspidariidae
Gastrochaenidae
Pharidae
Hiatellidae
Donacidae
Semelidae
Sphaeriidae
Chamidae
Cardiidae
Petricolidae
Glossidae
Veneridae
Corbiculidae
Vesicomyidae
Arcticidae
Ungulinidae
Mactridae
Pholadidae
Corbulidae
Myidae
Dreissenidae
Teredinidae
Xylophagidae
Palaeoheterodonta
Pteriomorphia
Euheterodonta

Figure 12. A phylogenetic tree of the Bivalvia based on Giribet and Distel (2004, fig. 3.6), showing the occurrence of cephalic eyes (stars) and ectopic pallial eyes belonging to Categories 1 (Arcoida, Mytilidae, Pteriodea, Anomiodea), 2 (Limoidea, Pectinidae, Spondylidae) and 3 (Pandoroidea, Cardiidae, Myidae).

Fig. 6) to create a structure that (i) has a greater breadth of view and which could (ii) but only in theory, produce a 'summed' image (Dawkins 1997). The limopsid *Philobrya munita* has a pallial eye that seems to be intermediate between the inverted cup and compound ommatidial eyes in that like the latter it is everted although pigment and photosensory cells are not inter-mixed (Morton 1978).

As Morton (2001) pointed out, however, no bivalve has any kind of brain that could ever recreate within it an image, regardless of the sensory sophistication of the eye that receives the enhanced differences in light associated with such improvements. The paired optic lobes of the cerebral ganglia of *Pecten maximus* are relatively 'large' for a bivalve (see Dakin 1909, plate 6, fig. 28) but nowhere near as large as those of cephalopods, for example, *Octopus vulgaris* Cuvier, 1797 (see Wells 1968, fig. 9.7; Hanlon and Messenger 1996, fig. 2.8) and which possess some 65 million nerve cells (Young 1971) involved not only in visual analysis but also act as visual memory stores.

Moreover, the fact that some bivalves, notably representatives of the Arcoidea and Pectinidae, possess so many pallial eyes along the mantle margin suggests that the eyes are highly important. In what way? In the case of the Arcidae, the presence of many compound eyes may allow for the detection of movement, as each is stimulated in succession by a moving object (Nilsson 1994). The same probably applies to the parallel circlets of eyes along the left and right mantle margins of scallops.

However, because Category 1 eyes develop on the outer mantle fold under the shell and periostracum, what changes in light they detect must be of poor visual quality. They, thus, perceive only a passing shadow. A human eye with a lens cataract might provide an analogy. This is not so, however, in those bivalves that developed pallial eyes on either their middle (Category 2) or inner (Category 3) mantle folds since they develop beyond the shell and periostracum. The increased visual acuity associated with such 'external' locations has been enhanced further by increases in structural complexity. How might this have been achieved?

In the Limidae and Cardiidae, there are ciliated pallial tentacles or accessory ciliated sense organs, in the former and latter, respectively, located upon specialized sub-folds of the middle or inner mantle fold, again respectively. Representatives of those two only distantly related bivalve families with the most sophisticated retina-based eyes, that is, the Laternulidae and Pectinidae both possess a ciliary-based proximal retina (Barber and Land 1967, Bell and Mpitsos 1968). It seems possible, however, that invagination of either an adjacent ciliated sensory tentacle or accessory organ, respectively, and incorporation of such a structure into these eyes might explain how the ciliary-based distal retina has been developed in both of them (as illustrated in Figs. 7 and 10, respectively). This would further explain how the double retina eyes of representatives of both families have evolved. They are, hence, also, an example of convergent evolution.

It seems possible, therefore, that the duplication of structures, in these cases either sensory tentacles or accessory organs, opens up the potential not only for new structures to evolve but also to alter and enhance functions. Hence, the original proximal retina was and still is responsible for detecting changes in light intensity and thus stimulating the shadow reflex. However, the distal retina, derived from an invaginated ciliated accessory structure, provides the **potential** for image formation and the detection of movement in representatives of the Pectinidae and Laternulidae. In the absence of optic lobes capable of synthesizing such information, however, these complex eyes must await matching cerebral sophistication.

The Arthropoda provides a well-known example of how the duplication of appendages has facilitated not just ambulatory but also mandibular diversification resulting in the extraordinary adaptive radiation of the phylum's numerous representatives and, hence, also of their eyes (Oakley 2003). In the Bivalvia, pallial fold duplication has resulted in improvements to the total array of peripheral pallial senses, most notably, however, the visual one. Improvements to the pallial visual senses have, however, occurred at different times in different phylogenies and on different components of the mantle margin. This has been achieved through (i) selective gene-induced ectopism; (ii) pigment-cup evagination in Category 1 eyes; (iii) invagination of accessory structures in some eye Categories 2 and 3 to form a distal retina in representatives of the Pectinidae and Laternulidae; and (iv) natural selection.

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Understanding the cephalic eyes of pulmonate gastropods: A review*

Marina V. Zieger and Victor Benno Meyer-Rochow

Jacobs University Bremen, School of Engineering and Science, Research II, Camps Ring 6, D-28759 Bremen, Germany,
marinazi@umich.edu

Abstract: This review showcases one group of gastropod's ability to perceive light through the eyes. The central question is simple: what are the visual performances and tasks of cephalic eyes in gastropods? That topic in itself is rather broad and is here applied to pulmonate gastropods, coming from terrestrial and aquatic biomes as well as different habitats and microhabitats, exhibiting different life-styles and light-tolerances. Therefore, the main objectives have been to analyze (1) anatomical and ultrastructural eye characteristics, (2) optical systems, (3) image-forming capabilities and possible functional consequences of eye size and design, (4) interactions between gastropods and their environment mediated by the visual information obtained through the eye, and (5) the specific visual tasks that the eyes serve. During the course of this study, a range of variations (= adaptations) in both optical and retinal design parameters, including eye size, aperture size, quality of optical image, retinal shape, sampling density, and optical sensitivity were discovered. All species of pulmonate gastropods studied have paired simple camera-type eyes that operate with advanced fixed focal-length optics. However, in terrestrial snails and slugs as well as freshwater limpets, the optics cannot produce a focused image on their shallow retinas. This seems to indicate that eyes in these species are not designed to receive a focused image and are likely to measure only the average light intensity or quality over large angles rather than resolve fine image details. The aquatic snails examined are able to focus a sharp image on the photoreceptive layer of the retina due to the deepenings of the latter (at least in a localized region). Although there is a significant correlation between specialization of the eye (*e.g.*, quality of optical image, sensitivity, and resolution) for a particular visual task in a specific habitat that the species encounters, there is no correlation between cellular composition of the retina and light/dark preferences. Their high optical sensitivity allows terrestrial snails to perform the necessary visual tasks in both bright and dim light, whereas the eye in aquatic species functions preferentially under bright light conditions. In conclusion, pulmonate gastropods use their eyes primarily for the following two kinds of visual tasks: (1) discriminating objects and possible enemies in their environment and (2) monitoring the environmental brightness level to orient towards dark places. The first type of visual task is characteristic of the aquatic snails and is served by image-forming eyes; the second is typical of terrestrial snails and slugs and is best served by a blurred image. Attention is given to visual ecological adaptations, specific visual needs, and the evolutionary history of gastropods.

Key words: vision, eye optics, visual behavior, retina, molluscs

The literature dealing with invertebrate eyes is extensive and especially with regard to molluscs, includes excellent reviews on photoreceptor evolution (Eakin 1968, Salvini-Plawen and Mayer 1977, Vanfleteren 1982), comparative anatomy and optics (Land 1981, 1984, Land and Fernald 1992), and phototransduction and physiological mechanisms (Messenger 1981, 1991, Nasi *et al.* 2000). The most recent books by Land and Nilsson (2002) and Warrant and Nilsson (2006) represent a comprehensive account of all known types of eye and provide an up-to-date synthesis of our current knowledge of invertebrate vision. However, even in these latest publications, pulmonate lineages, reviewed more than 30 years ago by Kerkut and Walker (1975), are somewhat under-represented. Moreover, most of the conclusions on the importance of eyes and vision in pulmonates stem from just a handful of species, and of them the terrestrial snails belonging to the genus *Cornu* (Born, 1778) alone have attracted the bulk of attention. This, no doubt, was the result of the sustained, detailed, and elegant anatomical and ultrastructural examinations of the *Cornu aspersum aspersum* (Müller, 1774) eye by Eakin and Brandenburger and their colleagues (*e.g.*, Eakin and Brandenburger 1967a, 1967b, 1970, 1975a, 1975b, 1982, Brandenburger and Eakin 1970, Eakin and Ferlatte 1973, Brandenburger 1975, Brandenburger *et al.* 1976) as well as some early electrophysiological studies by Berg and Schneider (1967), Gillary (1970), Pasic *et al.* (1974), and Berg (1978). The present review deals with the cephalic eyes of gastropods generally, and in pulmonates in particular, focuses on eyes of various sizes, differences in retinal design, and the extent to which their optical equipment permits image formation. Some of our recent observations, together with those of other researchers, will be briefly summarized.

For more than fifteen years we have been studying the eyes of pulmonate gastropods, and in this paper we shall review what is known of the morphology and ultrastructure of their retinal cells. We shall discuss recent comparative anatomical and ultrastructural findings and results based on behavioral tests and investigations that have involved optical modeling. We will evaluate some of the new ideas that arose from analyses of morphological and ecophysiological adaptations to terrestrial and aquatic ways of life and will give possible reasons for the large variety of retinal designs and optical systems one encounters in the pulmonate eye. Special emphasis is given to the research of the authors and their associates.

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ANATOMY

With the exception of a few subterranean stylommatophoran forms (*e.g.*, *Cecilioides acicula* (Müller, 1774) and *Helicodiscus singleyanus* (Pilsbry, 1889) as well as some blind *Ellobiacea* (Grassé 1948), all pulmonates have a single pair of eyes located on the head. The position of the eyes in relation to the tentacles is frequently employed as a taxonomic criterion. In stylommatophoran land snails and slugs, as well as in systellommatophoran marine slug-like pulmonates, eyes are developed at the tips of the mobile, retractile tentacles. In freshwater basommatophoran snails and limpets, the eyes are located medially or laterally at the base of a single pair of mobile tentacles close to the cerebral ganglion. Each cephalic eye consists of a cornea, a lens, a vitreous body, a non-inverted retina, the eye capsule, and the optic nerve.

The plane of the bilateral symmetry of the cephalic eyes coincides with the dorso-ventral plane of the heads in *Lymnaea stagnalis* (Linnaeus, 1758), *Radix peregra* (Linnaeus, 1758), *Planorbarius corneus* (Linnaeus, 1758), and *Physa fontinalis* (Linnaeus, 1758) and with the ventro-lateral plane in species with an accessory retina; no preferred plane is demonstrable in the remainder of the terrestrial pulmonates.

Integument

In all of the investigated pulmonates the eyes are placed under a circular area of a specialized region of the integument, that at this location is thin, unpigmented, dome-shaped, and translucent. The function of the integument layer may be a protective one, reducing possible mechanical injury and desiccation, the latter presumably of importance for terrestrial and some aquatic molluscs that lead moderately amphibious lifestyles. The integument may also be involved in regulating osmotic and ionic processes, since aquatic species in particular have to continually osmoregulate, while terrestrial gastropods need to minimize water loss.

Perioptic sinus

Willem (1892) described a spacious blood lacuna, the perioptic sinus, from the region around the eye of *Lymnaea stagnalis*. Later, Stoll (1973) confirmed Willem's observations in the same species and Bobkova *et al.* (2004a) described the perioptic sinus in *Radix peregra, Physa fontinalis,* and *Planorbarius corneus*. The observations showed that the sinus is anatomically isolated from the rest of the hemocoel and connected to it only through the interstitial tissue.

It is well known that sinuses, located in strategic regions, may become cavities of the hydrostatic skeleton, with blood serving as the hydrostatic fluid. In terrestrial species, however, the situation is different; the blood cavity in the retractable optic tentacles together with the hemocoel may function as a hydraulic rather than a hydrostatic system. We suggested (Bobkova *et al.* 2004a) that freshwater basommatophora actively place their eyes into the perioptic sinus, firstly, in order to fix their rigidly-held eyes in a particular position with regard to the body, and, secondly, to minimize the risk of possible mechanical compression when the animal withdraws its body into the shell. However, limpets like *Latia neritoides* (Gray, 1850) and *Ancylus fluviatilis* (Müller, 1774) apparently do not have the perioptic sinus (Meyer-Rochow and Bobkova 2001), but at the same time, these species have cap-like shells and do not have to compress their body as much as, for example, *Lymnaea stagnalis*.

Basal lamina and eye capsule

The retinal cells rest on a basal lamina. The lamina separates the retina and eye capsule as well as the cornea and interstitial tissue or, as in some aquatic species, cornea and lumen of the perioptic sinus. The basal lamina is continuous throughout its length and of constant thickness. Both the basal lamina and eye capsule continue into branches of the optic nerve. Along the course of the nerve, the capsule is then gradually replaced by a sheath of glial cells as, for example, in *Lymnaea stagnalis* (Bobkova 1998).

The eye capsule consists mainly of striated collagen fibrils and a layer of muscle cells. The muscle cells are embedded circumferentially (except for the area of the cornea) into an amorphous matrix to which they are attached by hemidesmosomes (*e.g.*, *Cornu aspersum aspersum*: Eakin and Brandenburger 1972; *Lymnaea stagnalis*: Bobkova 1998). The muscles are innervated by fine neurites, containing cored vesicles. The source of these neurites has still not been clearly determined. Recently it was demonstrated by immunohistological means that the eye capsule receives serotonergic innervation (Zhukov and Tuchina 2008).

Retinal design

As a rule, the eyes of all pulmonates studied to date possess conventional, cup-shaped retinas (*e.g.*, *Cepaea nemoralis* (Linnaeus, 1758) and *Trichia hispida* (Linnaeus, 1758): Fig. 1A-B). The eyes of some freshwater pulmonates such as *Lymnaea stagnalis, Radix peregra, Physa fontinalis,* and *Planorbarius corneus* have retinas that are portioned into

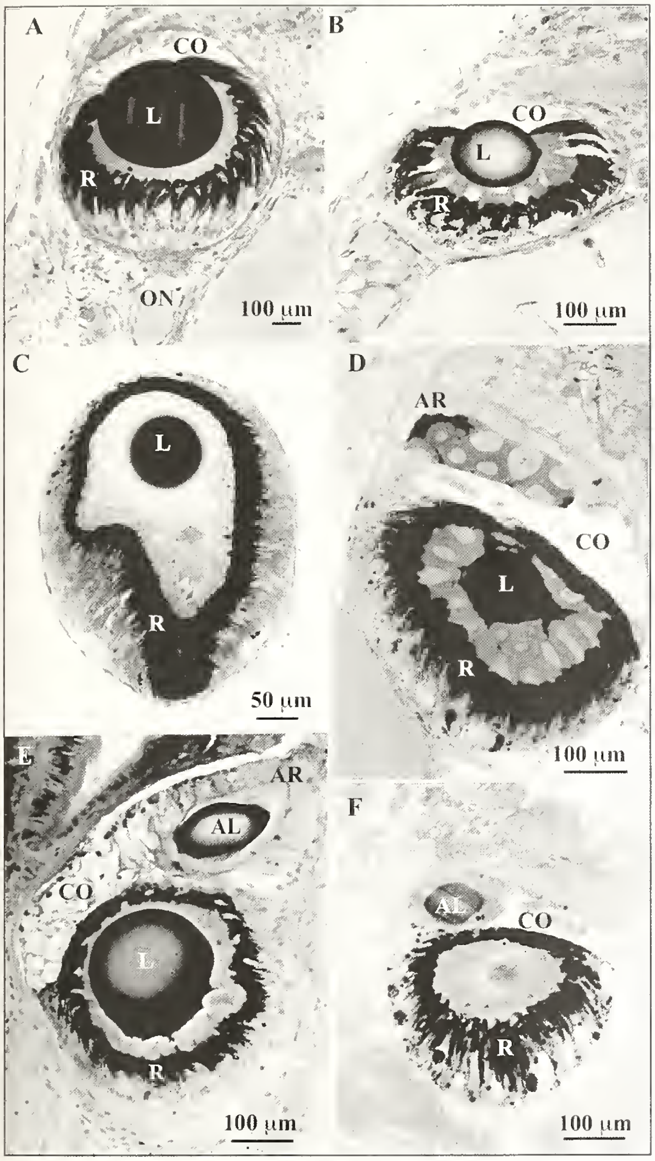

Figure 1. Light micrographs of longitudinal sections of camera-type eyes of various designs in pulmonate gastropods. (A) *Cepaea nemoralis*, (B) *Trichia hispida*, (C) *Lymnaea stagnalis*, (D) *Deroceras agreste*, (E) *Achatina fulica*, and (F) section through lens of additional eye in *Deroceras agreste*. AL, additional lens; AR, additional retina; CO, cornea; L, lens; ON optic nerve; R, retina. A, B, and C from Bobkova *et al.* 2004a; D and F from Bobkova *et al.* 2004b with permission of John Wiley and Sons, Inc.

dorsal and ventral depressions (termed "pits") (Fig. 1C). The pits are separated by an internal ridge, called a "crest", and based on their pigmentation, can be seen *in vivo* (Bobkova *et al.* 2004a). Of all non-pulmonate gastropods, only the opisthobranch *Navanax inermis* (Cooper, 1863) possesses an eye with a "bi-lobed" lens and a non-hemispherical retina (Eskin and Harcombe 1977). However, the question of whether this lens can produce an image on the retina has not been examined. Four distinct retinal layers in the eyes of gastropods can be distinguished, namely the microvillar, the pigmented, the somatic, and the plexiform layer.

Additional photoreceptive structures

Some pulmonates have a double eye. The additional photosensory organ was first described by Henchman (1897) in the slug *Limax maximus* (Linnaeus, 1758). The terrestrial snail *Achatina fulica* (Férussac, 1821) also has an additional retina (termed "accessory" by Tamamaki and Kawai 1983), invariably equipped with its own lens and an anatomically separate retina from that of the main eye (Fig. 1E). In *Limax flavus* (Linnaeus, 1758), Tamamaki (1989) found traces of a lens and a discontinuity of the two optic cavities. Therefore, we suggest calling the structure in question "an additional eye" rather than an "additional retina" or "accessory retina". Moreover, we have demonstrated that the cornea and the additional eye with its own nerve in the snail *A. fulica* may regenerate separately from the main eye (Bobkova *et al.* 2004b). But what we do not know is if such a regenerated structure is a functional organ.

The slug *Agriolimax reticulatus* (Müller, 1774) (Newell and Newell 1968) possesses an additional retina but lacks the additional lens, while *Deroceras agreste* (Linnaeus, 1758), also a slug, possesses both an additional retina and an extra lens (Fig. 1D, 1F). The additional lens can easily be isolated from its cavity. The lens is irregularly shaped and the vitreous body of the additional retina is continuous with the vitreous body of the main eye in *Deroceras agreste* (Zieger *et al.* 2008). Thus, we accept the term "additional retina" for species of pulmonates in which a vitreous body, associated with the additional retina, is continuous with vitreous body of the main eye, but we use the designation of "additional eye" when a discontinuity of the two optic cavities is present.

On the basis of the work by Tamamaki and Kawai (1983) on the eye of *Achatina fulica*, Newell and Newell (1968) on *Agriolimax reticulatus*, Tamamaki (1989) on *Limax flavus*, and us on *Deroceras agreste* (Zieger *et al.* 2008), it has become clear that the cellular composition of the additional retina is similar to that of the main eye. However, there are no screening pigment granules in the supportive cells of the additional retina. Neurosecretory cells were absent as well. The photoreceptor cells in the additional retina are large and their dome-shaped apices bear well-organized (regular), long microvilli. Accumulations of photic vesicles are also present, but the latter are not as tightly packed as those of the photoreceptors of the main retina. The microvilli were observed to be aligned in perpendicular orientations (Fig. 2), but we could not provide any morphological evidence for the view that the perpendicularly oriented mi-

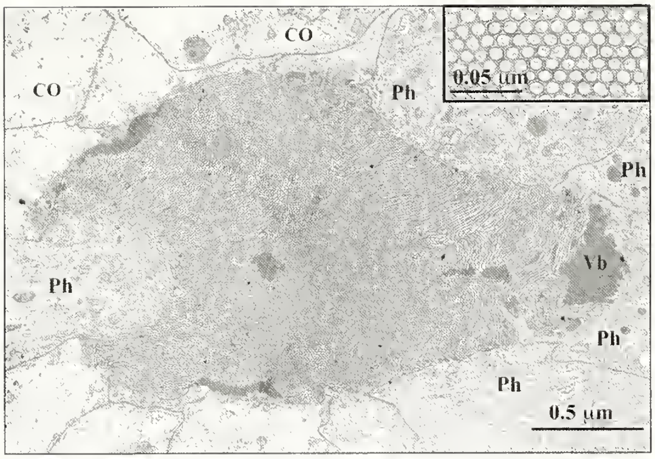

Figure 2. Electron micrograph of additional retina in *Deroceras agreste*. The inset shows a transverse section through the presumably light-sensitive microvilli. CO, corneal cells; Ph, photoreceptor cells; Vb, vitreous body. From Zieger *et al.* 2008 with permission of John Wiley and Sons, Inc.

crovilli might belong to neighboring (different) photoreceptor cells (Zieger *et al.* 2008).

As to the function of the additional retina, two theories have been proposed: (1) perception of changes in the intensity of the ambient light and (2) perception of infrared radiation. The latter could not be confirmed experimentally, but it has not been possible either to demonstrate that the additional eye is a visual organ at all. Moreover, the lack of screening pigment permits light to reach the presumed photoreceptor cells from any direction, making image formation impossible. However, Newell and Newel (1968), observing the behavior of *Agriolimax reticulatus* in a state with partially retracted optic tentacles, concluded that the additional eye would become exposed to light when the aperture of the main eye becomes fully masked by the pigmented integument. Therefore, the additional eye may be useful in situations like the partially retracted tentacle (Tamamaki 1989).

The hypothesis that in *Deroceras agreste* the additional retina could play a role as a sensor of polarized light had to be rejected as a consequence of carefully conducted behavioral tests (Vakoliuk 2005). Although we did not evaluate the optical system of the additional retina in *D. agreste*, we are nevertheless convinced, for reasons explained above, that it cannot form an image, even if it should turn out to be a functional light sensor.

The plexiform layer

The plexiform layer in all pulmonates studied to date is formed by axons of both photoreceptive and neurosecretory cell types (*e.g.*, Brandenburger 1975, Eakin and Brandenburger 1975b, Kataoka 1975, Eakin *et al.* 1980, Katagiri *et al.* 1995, Bobkova 1998, Meyer-Rochow and Bobkova 2001, Bobkova *et al.* 2004a). As shown for *Lymnaea stagnalis* (Bobkova 1998), the axons of the photoreceptor cells may be joined by synapse-like contacts of two morphological types. The first we call "invagination", for in this type the membrane of one axon drives a narrow finger-like protrusion into the other axon. Such contact seems to be very effective, as in the case of sensory cells in vertebrate electroreceptors of the "Lorenzini Ampoule" in skates (Byzov 1994). The second inter-axonal contact type in gastropods belongs to the flat "*en passant*" type.

The ultrastructure of synapses in the plexiform layer of the eye in *Lymnaea stagnalis* appears similar to the structure in the gastropod central nervous system. From studies in the snail *Achatina fulica*, it appears that there are at least two morphological types of synapses: (1) asymmetrical, or polarized, synapses with vesicle aggregation on one side of the active zone and (2) symmetrical synapses, with vesicle aggregations and pre-membranous densifications present on both sides of the synaptic cleft, thus indicating bi-directional transmission across the junction (cf., review by Chase 2002).

We suggest that contacts of both types can possibly provide summation of signals from groups of neighboring photoreceptors. Summation increases the number of photons received per channel during periods of low luminance. Such a strategy is employed by several other animals but only at the expense of spatial resolution (Seyer *et al.* 1998).

Organelles of great interest in the eye of *Lymnaea stagnalis* are the numerous small, clear, and dense vesicles. They are abundant close to the photoreceptor axonal membrane in the extracellular space (Bobkova 1998). Morphologically identical pictures of them were taken from the extracellular space in the CNS of *Cornu aspersum aspersum* (Chalazonitis 1971) where such vesicles were associated with exchanges of macromolecules between adjacent neuronal axons, axons and glial cells, and neurons and axons. We presume that the axons of the photoreceptor and possibly neurosecretory cells may have a metabolic link at the level of the plexiform layer. This process might be related to cell respiration because vesicles have been observed only in the peri-membrane portion of the axoplasm and in the intercellular spaces. The osmiophilic material in the vesicles can be one of the metabolic products of respiratory pigments like hemocyanin, which is known to function extracellularly.

Connections to the central nervous system

The axons of the retinal photoreceptors pass out of the eye by way of the optic nerve and form connections with second order neurons in the cerebral ganglion of the central nervous system. As demonstrated for *Lymnaea stagnalis* and *Planorbarius corneus*, afferent information from the eyes is widely distributed throughout the CNS. Nerve fibers, starting in the ipsilateral optic nerve were traced through the cerebral ganglion to the contralateral optic nerve, suggesting tight interactions between the two eyes (Zhukov and Tuchina 2008). Optic tract projections were found to end on the hair cells of the statocyst in *Lymnaea stagnalis*. The animal initially responds to light with phototactic behavior and moves towards the light, but in response to mechanical turbulence it clings to the surface. Such paired visuo-vestibular conditioning results in conditioned escape behaviors (cf., review by Sakakibara 2006).

Efferent innervation of the eyes occurs as well. Efferent fibers from the CNS form a varicose plexus within the eye capsule in *Lymnaea stagnalis* and *Planorbarius corneus* (Zhukov and Tuchina 2008). Serotonergic efferent fibers influence the amplitude of the electroretinogram and change absolute sensitivity to light in *Lymnaea stagnalis* (Zhukov *et al.* 2006).

OPTICAL SYSTEM

The optics of the eyes of gastropod molluscs vary greatly and range from a pigmented pit without a lens to sophisticated eyes with hard spherical lenses and image-forming capacity (Land 1984). According to Messenger (1981), the advanced cephalic eyes present in pulmonates can be classified as a "closed vesicle, camera-type eye", capable of forming an image on the retina (Nilsson 1989). However, such a categorization suggests a certain degree of homogeneity within the taxon but provides little information on possible modifications and/or variations of the optical system as well as the visual adaptations seen in gastropods.

Based on numerous pulmonate species examined by us (Bobkova *et al.* 2004a, 2004b), we now conclude that the positions or shapes of the lenses have evolved to become optimized in different environments as a response to the prevailing light conditions. Muscular or other connective tissues attached to the lens were not seen, and no kind of membrane or sheath delineating the lens appears to have developed. For the terrestrial snail *Cornu aspersum aspersum* it has been suggested that the shape of the eye could be changed through the actions of the musculature of the eye capsule and associated capsular strands (Mortensen and Eakin 1974). However, no experimental proof for this view has ever been presented. Based on our own observations, Bobkova *et al.* (2004a) concluded that pulmonates have a fixed focal-length optical system and a total absence of accommodative ability. The main components of the optical system in all of the pulmonates studied until now are the cornea and the lens.

Cornea

The convex-concave cornea represents the most anterior part of the eye vesicle. In terrestrial snails it is much thicker than that of the aquatic species (*e.g.*, in molluscs with comparable eye sizes like *Trichia hispida* and *Radix peregra*, the thickness of the cornea at its center is about 13 and 3 μm, respectively) (Bobkova *et al.* 2004a). Yet, despite the difference, comparisons of the corneae of the eyes of terrestrial (*e.g.*, the slug *Limax flavus*: Kataoka 1977, the snail *Achatina fulica*: Bobkova *et al.* 2004b) and aquatic pulmonates (*e.g.*, the snail *Lymnaea stagnalis*: Stoll 1973, Bobkova 1998, the slug *Onchidium verruculatum* (Cuvier, 1830): Katagiri and Katagiri 1998, the freshwater limpets *Ancylus fluviatilis* and *Latia neritoides*: Meyer-Rochow and Bobkova 2001) have revealed that the corneae are structurally similar to each other (Fig. 3A-B). We believe that the difference in thicknesses reflects the degree to which protection of the eye, for example in terrestrial snails against desiccation, is at a premium.

The cornea is composed of a monolayer of elongated, flattened, and *in vivo* transparent cells, reaching from the basal lamina to the optical cavity. The cells in aquatic species are strongly interdigitating. The inner surface of the corneal cells forms short irregular microvilli that are oriented toward the lens. The nuclei are situated in the basal cytoplasm. The flaky but electron-translucent cytoplasm of the corneal cells contains a few mitochondria, glycogen particles, rough endoplasmic reticulum, free ribosomes, and, directed toward the vitreous body, secretory vesicles filled with electron-dense granular material (Fig. 3A-B). The corneal cells are laterally connected with each other and with the most anterior retinal cells at the end of the cornea by septate junctions and *zonulae adhaerentes*. Basally they are anchored to the basal lamina by hemi-desmosomes. Corneal cells, together with retinal supportive cells, are known to contain secretory vesicles. The electron-dense granular content of the vesicles closely resembles that of the vitreous body and the lens.

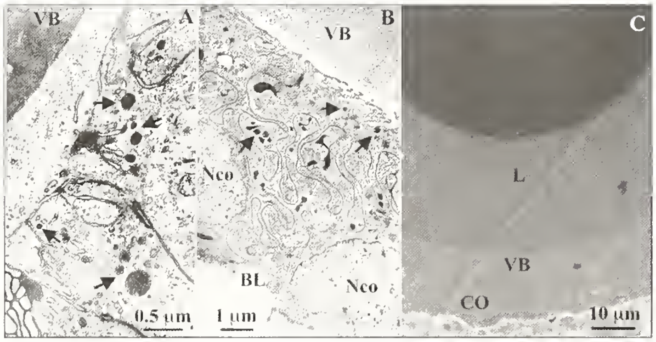

Figure 3. Electron micrographs of approximately longitudinal sections through the cornea: (A) *Deroceras agreste*, (B) *Radix peregra* and section through lens and cornea in (C) *Lymnaea stagnalis*. CO, cornea; L, lens; Nco, nucleus of corneal cell; VB, vitreous body; Arrows, secretory vesicles.

Therefore, the corneal cells are thought to be involved in the genesis of lens material (*e.g.*, in *Cornu aspersum aspersum*: Eakin and Brandenburger 1967a; *Limax flavus*: Kataoka 1977; *Lymnaea stagnalis*: Stoll 1973, Bobkova 1998; *Arion rufus* (Linnaeus, 1758); and *Deroceras agreste*: Zieger *et al.* 2008). Blumer (1995) described the transport of electron-dense material toward the lumen in juvenile marine non-pulmonate gastropods.

In an aquatic environment the cornea is not very useful as a refracting interface, and any necessary refraction therefore depends on the properties of the lens alone. Obviously, we have to be mindful of the fact that the cornea, if in air, shortens the focal length of the optical system, but even so, it has earlier been shown that in the terrestrial snails *Cepaea nemoralis* and *Trichia hispida* only the lenses play a significant role as the refractory surfaces (Gál *et al.* 2004). We therefore assume that the lens is the main element of the optical system not just in the aquatic, but in terrestrial species as well.

Lens

Gastropod eyes possess an "acellular" lens (Eakin 1972) and a vitreous body that shares certain features with the lens. Freshwater snails like *Lymnaea stagnalis*, *Radix peregra*, *Physa fontinalis*, and limpets like *Ancylus fluviatilis* and *Latia neritoides* have spherical lenses. However, all of the studied terrestrial snails and slugs (Newell and Newell 1968, Brandenburger 1975, Eakin *et al.* 1980, Meyer-Rochow and Bobkova 2001, Bobkova *et al.* 2004a, Zieger *et al.* 2008) as well as the marine slug *Onchidium verruculatum* (Katagiri and Katagiri 1998) have ovoid lenses of lamellar substructure with optical axes either along or across their ovoid outlines. Spherical lenses of the aquatic species are usually hard enough to permit their surgical removal from the eye for further study. The ovoid lenses of the terrestrial snails and slugs are also hard (Bobkova *et al.* 2004a), but some stylommatophoran snails, like *Strophochelius* sp. (Pfeiffer, 1842), possess a jelly-like lens that tears easily during dissection (Oswaldo-Cruz and Bernardes 1982).

The spherical lenses of most aquatic and terrestrial species can easily be isolated from the retinal cavity, but it is practically impossible to separate the non-spherical lenses (Zhukov *et al.* 2002) from the vitreous body of the freshwater pulmonate snail *Planorbarius corneus* (Bobkova *et al.* 2004a), suggesting intimate connections and possibly a common origin of lens and vitreous body.

To keep the size of the eye sufficiently small, the focal length needs to be kept short in relation to the size of the lens. This requires the lens to be more or less spherical. However, a homogeneous and spherical lens would suffer from serious spherical abberations, in which rays off axis are refracted too severely, so that a sharp image cannot be obtained and an undesirably large blurred circle is created instead. Another problem is that the lens would have rather a long focal length (Land and Nilsson 2002).

Matthiessen (1886) showed that both these problems could be overcome if the lenses were optically inhomogeneous with a gradient of higher to lower refractive index from center to periphery. Then, rays entering the lens could be bent continuously within the lens through refraction and not just at the lens's outer and inner surfaces. A lens design like this would effectively increase the power of the lens and shorten its focal length, correct spherical aberration, and, thus, reduce or even abolish the deleterious effects of an optically homogeneous lens (Land 1984).

As a rule, unstained transverse sections through a snail's lens sometimes reveal regular concentric layers that are discernible under both light and electron microscopes (*e.g.*, the marine slug *Onchidium verruculatum*: Katagiri and Katagiri 1998; freshwater and terrestrial snails and slugs: Bobkova *et al.* 2004a, Zieger *et al.* 2008; and freshwater limpets: Meyer-Rochow and Bobkova 2001) (Fig. 3C). A heterogeneous staining pattern can furthermore indicate an optically inhomogeneous construction of the lens (Hamilton *et al.* 1983, Bobkova *et al.* 2004a).

Demian and Yousif (1975) demonstrated that the lens, laid down in the lumen of the eye, grows through the addition of concentric layers of secretions from the centre outwards. Thus, it seems that lenses with a radial gradient of refractive index should be relatively easy to build because proteins varying in optical density may be synthesized and laid down successively. However, the chemical composition of the acellular gastropod lenses remains enigmatic. The sea hare *Aplysia californica* (Cooper, 1863) is the only gastropod that has been examined for crystallins, *i.e.*, proteins known to be responsible for the optical properties of transparent lenses and corneae (Tomarev and Piatigorsky 1996).

Vitreous body

In order to have a potential for image formation, the lens and the retina need to be separated (Land 1981, Seyer 1994, Seyer *et al.* 1998, Gál *et al.* 2004). In the camera-type eyes, separation of the lens and retina is achieved by presence of a vitreous body. The vitreous body surrounds the lens and has contact with the receptive and non-receptive parts of the retina. The vitreous body and lens both contain grainy or tubular substructures, but those making up the vitreous body are always less electron-dense than those of the lens interior (Fig. 3C). The vitreous body is considerably less extensive in many terrestrial pulmonates, and larger in some freshwater pulmonates that demonstrate good visual abilities. The near lack of separation between the lens and the retina in most terrestrial pulmonates seems to indicate that their eyes are not built to receive a focused image.

The vitreous humor is continuous between the additional and the main retinae in *Agriolimax reticulatus* (Newell and Newell 1968) and in *Deroceras agreste* (Zieger *et al.* 2008).

ULTRASTRUCTURE OF THE RETINAL CELLS

Photoreceptors

With rare exceptions, *e.g.*, *Planorbarius corneus* (Zhukov *et al.* 2002), the majority of the species investigated have two morphologically distinct kinds of "rhabdomeric photoreceptors" (Eakin 1963). The first kind ("type I photoreceptors") is characterized by long microvilli and massive aggregations of so-called "photic vesicles" (Eakin 1990). The second photoreceptor type ("type II") is less common. It lacks the dense packing of photic vesicles and bears shorter microvilli. The type II photoreceptor has been described from the retinae of the terrestrial snails *Cornu aspersum aspersum* (Brandenburger 1975), *Succinea putris* (Linnaues, 1758) (Zunke 1979), *Strophochelius* sp. (Oswaldo-Cruz and Bernardes 1982), *Trichia hispida* (Bobkova *et al.* 2004a), the terrestrial slugs *Limax maximus, Ariolimax californicus* (Cooper, 1872) (Eakin and Brandenburger 1975b), *Athoracophorus bitentaculatus* (Quoy and Gaimard, 1832) (Eakin *et al.* 1980), *Limax flavus* (Kataoka 1975), and *Deroceras agreste* (Zieger *et al.* 2008). The marine slugs *Onchidium verruculatum* (Katagiri *et al.* 1995), and the freshwater basommatophoran snails *Lymnaea stagnalis* (Stoll 1973, Bobkova 1998), *Radix peregra,* and *Physa fontinalis* (Bobkova *et al.* 2004a) have the second photoreceptor type as well.

Photoreceptor cells with long microvilli may have a finger-like (as in freshwater species (Bobkova *et al.* 2004a) and the slug *Deroceras agreste* (Zieger *et al.* 2008)) (Fig. 4A-C), flared (as in *Arion rufus* (Zieger *et al.* 2008)) (Fig. 5) or short and dome-shaped apical portion as in *Ariolimax californicus* (Eakin and Brandenburger 1975b) and *Cepaea nemoralis* (Bobkova *et al.* 2004a). The short microvilli of the photoreceptors may be brush-like, whorled (as in the distal depression of the retina), or regularly arranged (Fig. 4D-E). Electrophysiological results suggest in *Limax flavus* that type I photoreceptors operate in dim and type II photoreceptors in bright light (Suzuki *et al.* 1979). However, we cannot exclude the possibility that the cells belong to the same functional type of photoreceptor and represent stages in the process of receptor cell renewal (death and replacement) in the mature retina. Moreover, based on electrophysiological recordings from a variety of gastropods (*e.g.*, Dennis 1967, Gillary and Wolbarsht 1967, Hughes 1970, Gillary 1974, Berg 1978, Suzuki *et al.* 1979, Zhukov and Gribakin 1990, Chernorizov *et al.* 1994), there is no convincing evidence for more than a single spectral sensitivity peak between 480 and 505 nm wavelength, suggesting that at least physiologically there is only one type of receptor.

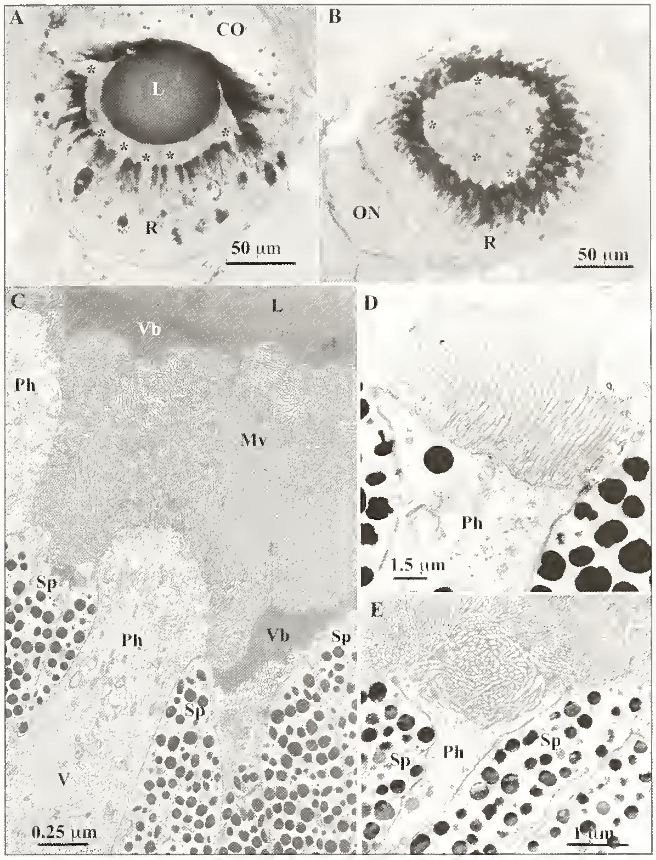

Figure 4. Light (A and B) and electron (C, D, and E) micrographs, featuring two morphological types of photoreceptor cells in pulmonates. Nearly longitudinal (A and C) and transverse (B) sections through the eye of *Deroceras agreste*, showing finger-like apices (asterisks). Photoreceptors with short and regularly arranged microvilli in *Lymnaea stagnalis* (D) and whorled microvilli in the distal depressions of the *Trichia hispida* retina (E) are discernible. CO, cornea; L, lens; Mv, light sensitive layer of microvilli; ON, optic nerve; Ph, photoreceptor cells; R, retinal cup; Sp, supportive (pigmented) cells; Vb, vitreous body. A, B, and C from Zieger *et al.* 2008; D and E from Bobkova *et al.* 2004a with permission of John Wiley and Sons, Inc.

As evident from *in vivo* observations and sections of fixed tissues, the retinas of all pulmonate snails are deeply pigmented. However, in spite of the presence of specialized supportive (or pigmented) cells, photoreceptor cells also contain screening pigment granules in freshwater snails (Bobkova 1998, Bobkova *et al.* 2004a) and limpets (Meyer-Rochow and Bobkova 2001) as well as in the terrestrial snails *Trichia hispida* (Bobkova *et al.* 2004a) and *Cornu aspersum aspersum* (Eakin and Brandenburger 1982) and the marine slug *Onchidium verruculatum* (Katagiri *et al.* 1995) (Fig. 6A-B). Photoreceptors in terrestrial slugs (Newell and Newell 1968, Eakin and Brandenburger 1975b, Kataoka 1975) and

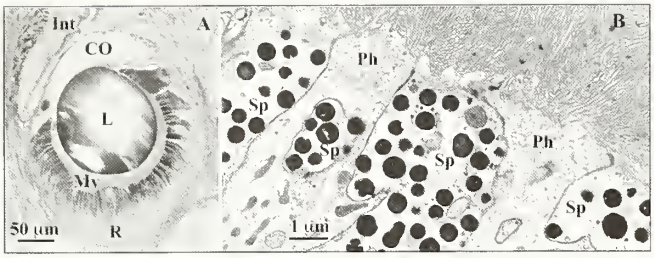

Figure 5. Light (A) and electron (B) micrographs of longitudinal sections through eye and apical portion of the retina of *Arion rufus* showing shallow retina (A) and flared apices of photoreceptor cells with long microvilli (B). CO, cornea; Int, integument; L, lens; Mv, microvillar layer; Ph, photoreceptor cells; Sp, pigmented supportive cells. A and B from Zieger *et al.* 2008 with permission of John Wiley and Sons, Inc.

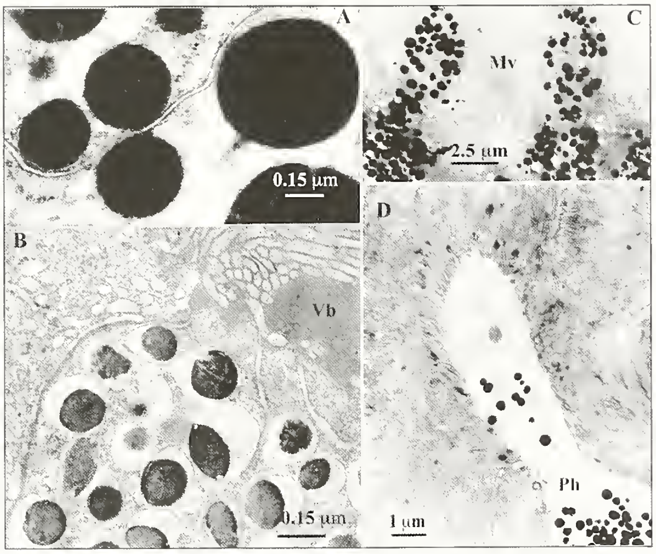

Figure 6. Electron micrographs, showing pigment granules in the supportive cells of *Arion rufus* (A) and pigmented granules, affected by bright light, in the photoreceptor cell of *Deroceras agreste* (B), with pigment granules filling the apices of the photoreceptor cells following deep light adaptation (C) and fewer pigment granules following deep dark adaptation (D) in the photoreceptor cells of *Lymnaea stagnalis*. Mv, microvilli; Ph, apex of photoreceptor cell; Vb, vitreous body. C and D from Bobkova *et al.* 2004a with permission of John Wiley and Sons, Inc.

the snail *Cepaea nemoralis* (Bobkova *et al.* 2004a) contain no screening pigment granules. A certain vertical light-dependent pigment granule displacement (often called "migration") was revealed in freshwater snails (Bobkova 1998, Zhukov *et al.* 2002, Bobkova *et al.* 2004a). In samples prepared for morphological investigations during periods of total light adaptation, we were able to observe that the finger-like apices became filled in with screening pigment granules. Conversely, pigment granule dispersion occurred when a light-adapted eye was exposed to darkness and, thus, indicates dark adaptation (Bobkova 1998) (Fig. 6C-D).

Our ultrastructural observations have shown that photoreceptor cell types I and II occur in close approximation to each other and are jointly present at the level of the retinal pigment layer in the eye of, for example, *Lymnaea stagnalis* (Bobkova 1998) and *Cepaea nemoralis* (Bobkova *et al.* 2004a). However, we never found any specialized mechanical or functional contacts between the two types of these cells.

Microvilli and photic vesicles

One of the most important selective pressures in the evolution of photoreceptors appears to have been the need to increase the area available for visual membranes—in other words, to lay the foundation for an increase in photosensitivity that depended on the maximization of space for photoreceptive membranes. Rhabdomeric photoreceptors achieve this by increasing the total number of microvilli. The small diameter of an individual microvillus, which is approx. 600 Å in the dark-adapted eye irrespective of the type of photoreceptor, helps in this regard but also poses a lower limit for miniaturization as the organization and dimensional characteristics of the microvilli appear to be similar in all of the invertebrate eyes investigated to date.

An axial cytoskeleton is present in the tubular lumen of the microvilli; it is formed by bundles of fibrils that run along the length of the microvilli and are connected to the membrane (Fig. 7A). Microvilli reach their highest degree of order during periods of dark adaptation but are found to enlarge ("swell up"), following brief exposures to bright light, or to become dramatically disorganized when an isolated eye is exposed to bright light for a longer period of time (Bobkova 1998) (Fig. 7B). The processes of microvillar disruptions by light in the snail eye are likely to be similar to the changes that bright light exposure causes in the compound eyes of crustaceans (reviewed by Meyer-Rochow 1994, 2001) and insects (Meyer-Rochow *et al.* 2002), where bright light is known to affect the axial cytoskeleton and the lipid fraction of the microvillar membrane itself (Kashiwagi *et al.* 1997, 2000).

Photic vesicles are important and characteristic cytoplasmic organelles in the eyes of gastropod molluscs (Fig. 7C). Whittle (1976) discussed the controversial origin of the photic vesicles, *i.e.*, of Golgi apparatus origin according to Eakin and Brandenburger (1967b) but vesiculations of the smooth endoplasmic reticulum according to Kataoka (1975), and concluded that the evidence is entirely consistent with reticular specializations. Eakin (1990) has summarized data on structure, origin, fate, and function of the

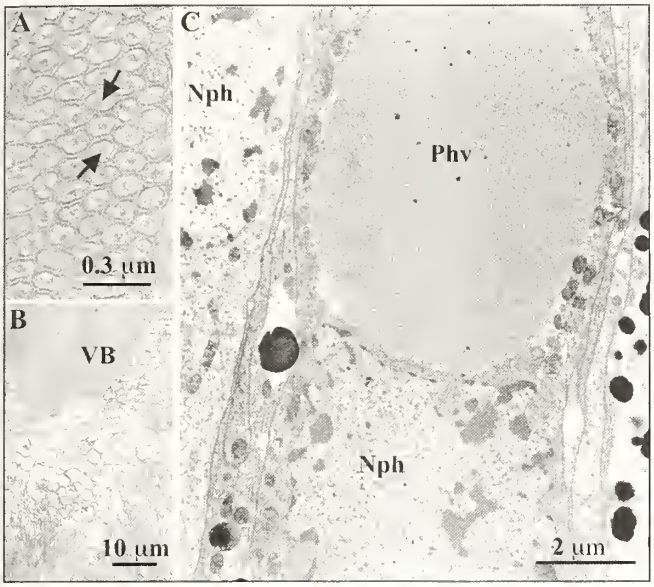

Figure 7. Electron micrographs, showing transverse section through microvilli (A) and microvillar layer affected by bright light in the eye of *Lymnaea stagnalis* (B) and aggregation of photic vesicles (C) in the perinuclear area of photoreceptor cells in *Cepaea nemoralis*. Arrows, axial cytoskeleton; Nph, nuclei of photoreceptor cells; Phv, photic vesicles; VB, vitreous body.

photic vesicles as "transporters of a photopigment retinochrome and calcium" and likened them to the lamellated bodies in cephalopod photoreceptors shown by Hara *et al.* (1967) to contain retinochrome. Later, fluorescent histochemical techniques, using a reducing agent, have shown that rhodopsin and retinochrome are present in the microvillar and somatic layers of the stalks of the eyes of the marine pulmonate slug *Onchidium verruculatum* (Katagiri *et al.* 2002) and the terrestrial slug *Limax flavus* (Ozaki *et al.* 1983).

Using antibodies against squid retinal proteins, Katagiri *et al.* (2001) determined the localization of three retinal proteins (rhodopsin, retinochrome, and retinal-binding protein), and thus demonstrated the presence of a rhodopsin-retinochrome system in the eyestalk of the marine pulmonate *Onchidium* sp. Later, Katagiri *et al.* (2002) confirmed by fluorescence histochemistry the presence of rhodopsin and retinochrome in specific regions of the retina. Accordingly, the visual pigment rhodopsin is present in the microvilli of the photoreceptive cells, while the photic vesicles themselves may be regarded as a store for retinaldehyde in the form of retinochrome-chromophore.

Rhodopsin and retinochrome function cooperate to mutually regenerate photopigment (Terakita *et al.* 1989). Upon illumination, rhodopsin is converted to metarhodopsin, and retinochrome to meta-retinochrome. The 11-cis-retinal in rhodopsin is photoisomerized to the all-trans configuration. Conversely, the all-trans-retinal in the retinochrome is photoisomerized to its 11-cis-isomer. The 11-cis-retinal is required for rhodopsin formation. In the dark, rhodopsin and retinochrome are regenerated from these two isomers by chromophore exchange.

Supportive pigmented cells

Supportive pigmented cells are located between the photoreceptor cells. Their apical portion bears very short membranous microvilli-like projections into the vitreous body. The cells form the border of the fixed pupil aperture. In freshwater snails, limpets, and some terrestrial species, supportive pigmented cells have column-like apices (*e.g.*, *Lymnaea stagnalis*: Stoll 1973, Zunke 1979; *Onchidium verruculatum*: Katagiri *et al.* 1995, Bobkova 1998; *Latia neritoides* and *Ancilus fluviatilis*: Meyer-Rochow and Bobkova 2001; *Planorbarius corneus*: Zhukov *et al.* 2002; *Radix peregra*, *Physa fontinalis*: Bobkova *et al.* 2004a). However, in some terrestrial slugs and snails, the supportive pigmented cells envelope the photoreceptors, send deep and multiple projections into the photoreceptor cytoplasm, and are crowded with innumerous pigment granules (*viz.*, *Helix pomatia*: Schwalbach *et al.* 1963, Röhlich and Török 1963; *Cornu aspersum aspersum*: Eakin and Brandenburger 1967a; *Limax flavus*: Kataoka 1975; *Ariolimax californicus*: Eakin and Brandenburger 1975b; *Achatina fulica*: Tamamaki and Kawai 1983; *Cepaea nemoralis*: Bobkova *et al.* 2004a; *Deroceras agreste* and *Arion rufus*: Zieger *et al.* 2008). However, in species with finger-like apices (cf., section on "Photoreceptors") they never penetrate into the spaces between the latter. In the nocturnal slug *Limax maximus*, lateral branches along the sides of the photoreceptor cells have been described to often lack pigment granules (Eakin and Brandenburger 1975b).

The nuclei of the supportive (pigmented) cells are located in the basal region of the somatic and plexiform layers. They are much smaller than the nuclei of the photoreceptor cells and are identifiable by their condensed heterochromatin (Fig. 8A). Multiple rings of rough endoplasmic reticulum together with large numbers of mitochondria are present in the perinuclear area, suggesting that the supportive pigmented cells are synthetically active retinal cells. The cells themselves are anchored by hemi-desmosomes to the basal lamina.

Although the chemical nature of the retinal screening pigment has not been analyzed in detail, generally it is assumed that the pigment is a form of the melanin (*e.g.*, *Lymnaea stagnalis*: Land 1968, Stoll 1973, Bobkova 1996, and *Cornu aspersum aspersum*: Eakin and Brandenburger 1967a, 1967b).

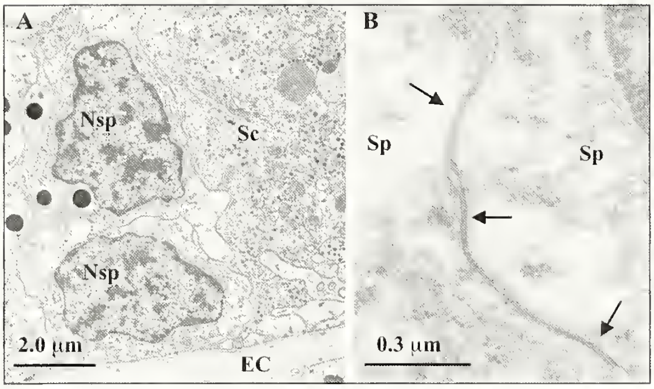

Figure 8. Electron micrographs, showing (A) nuclei of pigmented supportive cells (Nsp) and sites in (B) of gap junctions (arrows) between adjacent pigmented supportive cells (Sp) in *Arion rufus*. EC, eye capsule; Sc, neurosecretory cell. From Zieger *et al.* 2008 with permission of John Wiley and Sons, Inc.

Cellular contacts between adjacent cells

As a rule, and not restricted to molluscs, adjacent cells are connected with each other by mechanical cellular contact specializations like *zonulae adhaerentes*, located apically just above the septate junctions. The latter resemble those examined in the leech photoreceptors by Aschenbrenner and Waltz (1998) and are thought to contribute to the maintance of the cell polarity. Contacts of this nature are seen in the retinae of *Ariolimax californicus* (Eakin and Brandenburger 1975b), *Limax flavus* (Kataoka 1975), *Athoracophorus bitentaculatus* (Eakin *et al.* 1980), and *Lymnaea stagnalis* (Bobkova 1998). The existence of finger-like folds, projecting into adjacent cells, has been reported from the eyes of *Lymnaea stagnalis* by Bobkova (1998) and *Ariolimax californicus* by Eakin and Brandenburger (1975b). Similar cytoplasmic folding is known from vertebrate epithelial cells (*i.e.*, bronchial and intestinal kinds) to enable these cells to change their length. A property such as this, if it held true for the unusual mechanical contacts with folds, would agree with observations made by Stoll (1973) on light-dependent retinomotoric cell extensions and contractions in the retina of *Lymnaea stagnalis*.

The supportive cells (with heavily pigmented extensions, but only near their distal ends) form multiple gap-junctions between each other in *Lymnaea stagnalis* (Bobkova 1998) and *Arion rufus* (Zieger *et al.* 2008) (Fig. 8B). It appears as if this type of cell has a kind of syncytium-like intraretinal net.

Neurosecretory cells

The retinal cells that we termed 'neurosecretory' in *Arion rufus* (Zieger *et al.* 2008) are likely to belong to cells that are known in *Limax flavus* as "large ganglionic cells of the nervous system" (Kataoka 1975) and in *Cornu aspersum aspersum* (Brandenburger 1975), *Ariolimax reticulatus* (Newell and Newel 1968), and *Lymnaea stagnalis* (Stoll 1973, Bobkova 1998) as ganglion cells.

The cell types listed above are similar in appearance, contain a very large nucleus, and are endowed with prominent rough endoplasmic reticulum, numerous granulated osmiophilic dense-core and clear vesicles, and large liposomes or pigment-granule-like bodies, similar to the lipochondria of the *Aplysia* sp. ganglion cells described by Baur *et al.* (1977). Following Strumwasser *et al.* (1979), we have accepted the term "neurosecretory cells" for the cells in question in *Arion rufus*.

These large oval cells were found to form a single cluster in a delimited region of the eye of *Arion rufus* (Zieger *et al.* 2008) (Fig. 9A-C), and in *Trichia hispida* and *Cepaea nemoralis* (Zieger, unpubl. data). In the freshwater snails *Lymnaea stagnalis* (Bobkova 1998), *Radix peregra*, and *Physa fontinalis* (Zieger, unpubl. data), retinal cells of this type are diffusely distributed among other retinal cells. No synaptic contacts were found on the surface of the cell body of these cells in *A. rufus* (Zieger *et al.* 2008) and *Lymnaea stagnalis* (Bobkova 1998). However, Brandenburger (1975) demon-

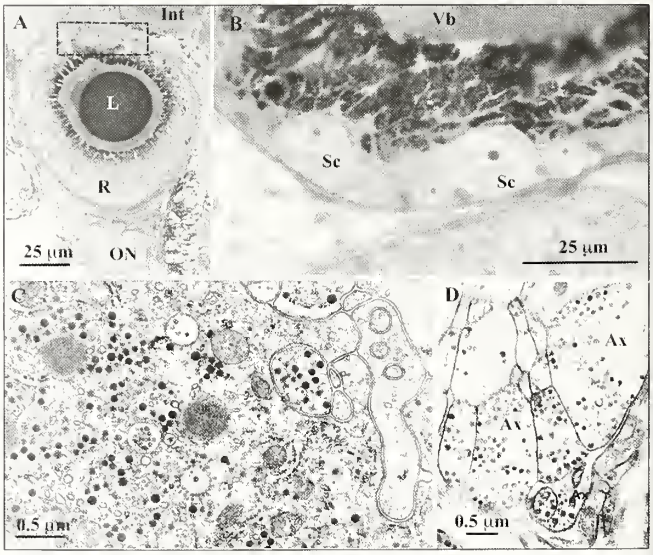

Figure 9. Light micrographs of tangential section (A) through cluster of neurosecretory cells (framed) and (B) enlargement of the cluster in the eye of *Arion rufus*. Electron micrographs of perinuclear region (C) and axons (D) containing dense-core vesicles in *Arion rufus*. Ax, axons; Int, integument; L, lens; R, retina; Sc, secretory cells; Vb, vitreous body. A and B from Zieger *et al.* 2008 with permission of John Wiley and Sons, Inc.

strated synaptic-like structures on the surface of the "ganglion" cells in *Cornu aspersum aspersum.*

The axons of the neurosecretory cells can be identified in the plexiform layer of the retina by their contents of dense core vesicles (Fig. 9D). We cannot completely rule out the possibility that the neurosecretory cells in the eye of *Arion rufus* are a kind of neuroendocrine cell type that releases its vesicular content directly into the body fluid to reach more distant effector organs.

As to the function of the retinal neurosecretory cells, we can only speculate that they could be involved in various aspects of the reproductive cycle (*i.e.*, gonadal development, sexual maturation, egg laying) or in some aspect of metabolic regulation. It was earlier shown that the retinal neurons in the marine non-pulmonate gastropods *Bulla gouldiana* (Pilsbry, 1895) and *Aplysia californica* are capable of generating a circadian periodicity. Actually, the cells are competent circadian retinal pacemakers and as in circadian pacemakers, the oscillators entrain to cycles of light and darkness, especially the 24-h light/dark cycle of the environment (cf., review by Whitmore and Block 1996).

In behavioural experiments with *Arion rufus*, it was shown that these slugs have a circadian rhythm (Lewis 1969). In fact, that light is highly effective in entraining circadian rhythmicity is also known for other gastropods like slugs (*Limax flavus*: Segal 1960; *Agriolimax reticulatus*: Newell and Newel 1968), terrestrial snails like *Helix pomatia* (Jeppersen 1977), *Cornu aspersum aspersum* (Bailey 1981), *Helix lucorum lucorum* (Linnaeus, 1758) (Flari and Lasaridou-Dimitriadou 1995), and *Achatina achatina* (Hodasi 1982) as well as the slugs *Limax pseudoflavus* (Evans, 1978) (Ford and Cook 1988, 1994), *Deroceras reticulatum* (Müller, 1774), and *Arion distinctus* (Mabille, 1868) (Hommary *et al.* 1998). However, the possession of an internal oscillator, which is capable of measuring the photoperiod has not been demonstrated in these pulmonate species. The notion that it is the retinal neurons, which express circadian rhythmicity themselves, has yet to be proven.

OPTICAL CALCULATIONS

In 1984 Hamilton and Winter pointed out that the degree of accuracy of the behavioral data on visual abilities can be checked by carrying out a resolution estimate based on the optical and structural characteristics of the eye. In other words, in order to know a snail eye's functional limitations, it is imperative to possess some information on the eye's optics and structural organization. Resolving power and sensitivity are key parameters in this context, and both can be approximated from anatomical and optical data. To determine the limitations of the optical system of the eyes in pulmonates, we followed methods (with some modifications: cf., Bobkova *et al.* 2004a, Gál *et al.* 2004) that were used earlier with considerable success in comparisons of eyes in some caenogastropods (Seyer 1992, 1994, Seyer *et al.* 1998). The methods described by Nilsson *et al.* (1988) and by Seyer (1992) were used to determine the focal lengths of freshly isolated lenses. The same methodological approach allowed us to compare optical systems in different pulmonate gastropods (Tables 1-3).

All of the pulmonate gastropod eyes investigated to date operate with fixed focal-length optics, which means that at least the principal focal length cannot be changed. The fixed focal-length optics design is described in some vertebrates (Walls 1942), who in order to accommodate to different distances adjust the position of the lens relative to the retina. However, in the absence of accommodation mechanisms in the eyes of gastropods, it appears that the almost infinite depth of focus characteristic of the eyes of aquatic gastropods makes accommodation unnecessary (Land 1981).

The shape of the gastropod eye lens was found to be spherical in aquatic species and elliptic, with an optical axis either across or along the lenticular ellipse, in terrestrial species. There are, however, exceptions: the lens of the aquatic snail *Planorbarius corneus* is rather more elliptic than spherical (Zhukov *et al.* 2002) and the terrestrial slug *Deroceras agreste* has a spherical lens (Zieger *et al.* 2008). The

Table 1. Comparative eye parameters of terrestrial pulmonates based on light and electron microscopy (in μm). Values are means.

	Arion rufus	*Deroceras agreste*	*Cepaea nemoralis*	*Trichia hispida*	*Agriolimax reticulatus*
Size of eyeball	240 × 290	220 × 220	312 × 320	142 × 220	140 × 180
Size of lens	150 × 200	110 × 110	152	81	130
Diameter of aperture	100	80	107	92	—
Center-to-center separation of receptors	5.6	17.0	21.0	15.0	6.0
*Receptor length	13.8	27.8	3.4-20.0	3.0-15.0	25.0-30.0
Authors	Zieger *et al.* (2008)		Bobkova *et al.* (2004)		Newell and Newell (1968)

* Values based on thickness of microvillar layer.

Table 2. Comparative eye parameters of aquatic pulmonates based on light and electron microscopy (in μm). Values are means.

	Ancylus fluviatilis	*Latia neritoides*	*Lymnaea stagnalis*	*Radix peregra*	*Physa fontinalis*	*Planorbarius corneus*
Size of eyeball	100 × 100	150 × 175	210 × 270	160 × 200	150 × 170	240 × 275
Size of lens	60	90	120	94	80	136
Diameter of aperture	43	63	60	73	65	140
Center-to-center separation of receptors	2.5	6.5	8.3	4.5	8.9	6.2
*Receptor length	7.0	31.0	71.0	18.0	49.1	18.5
Authors	Meyer-Rochow and Bobkova (2001)		Bobkova *et al.* (2004a)			

* Values based on thickness of microvillar layer.

Table 3. Comparative mathematically determined optical parameters in different species of pulmonate gastropods. Values are means; the symbol '—' denotes missing values.

Species	Principal focal length of the lens (μm)	Relative aperture	Matthiessen's ratio	Angular receptor spacing (degrees)	F-number	Diameter of Airy-disc (μm)	Resolving power (radian^{-1})	Sensitivity (μm^{2}sr^{-1})	Authors
Arion rufus	848	0.10	—	0.40	—	—	—	—	Zieger *et al.* (2008)
Deroceras agreste	207	0.40	3.7	4.70	2.5	3.00	—	—	Zieger *et al.* (2008)
Cepaea nemoralis	149	0.70	—	8.00	1.3	1.60	4.0	8.00	Gál *et al.* (2004)
Trichia hispida	122	0.80	—	13.00	1.2	1.50	2.5	4.00	Gál *et al.* (2004)
Agriolimax reticulatus	100	—	2.8	15.00	—	—	—	—	Newell and Newell (1968)
Lymnaea stagnalis	174	0.30	2.9	2.70-5.20	2.9	3.60	6.3-12.0	0.04-1.80	Gál *et al.* (2004)
Radix peregra	128	0.60	2.7	2.0-6.50	1.7	2.00	0.2-16.5	0.02-0.20	Gál *et al.* (2004)
Physa fontinalis	116	0.60	2.9	4.00-5.00	1.8	2.20	7.0-8.0	0.20	Gál *et al.* (2004)
Planorbarius corneus	241	0.60	3.5	2.50	1.8	2.00	13.0	1.40	Gál *et al.* (2004)

lenses in all species studied presumably possess a radial gradient of refractive index (*i.e.*, are optically in-homogeneous) irrespective of lens shape.

It is generally assumed that terrestrial animals cannot exploit the advantages of a spherical graded index lens. Instead, by having a curved outer surface on the cornea, they must make use of the low refractive index of air (1.0). Because the cornea then provides much of the refracting power, an interior lens can be designed specifically, for example, to correct spherical aberration (Land 1984, Nilsson 1989).

However, on the basis of our calculations it appears that the integument-cornea lens complex in the terrestrial snails *Cepaea nemoralis* and *Trichia hispida* is optically much less important than the refractive power of the lens alone (Gál *et al.* 2004). A similar assessment, not based on any calculations, was made for the slugs *Arion rufus* and *Deroceras agreste* (Zieger *et al.* 2008). For accuracy, we have made the essential calculations, and have included the mean values obtained into this review (see below).

The focal length of the system of two lenses (f_S) can be calculated as:

$$1/f_S = 1/f_L + 1/f_C - x/f_L f_C,$$

where f_L is principal focal length of the lens, f_C is focal length of the integument-cornea complex and x is the distance between the two lenses centers.

The focal length of the integument-cornea complex can be calculated as described by Gál *et al.* (2004).

Thus, in *Cepaea nemoralis* the focal length of the lens alone is 149 μm, and the focal length of the system composed of the two lenses (integument-cornea plus lens) is 144 μm. The corresponding figures are 122 and 113 μm for *Trichia hispida*, 207 and 170 μm for *Deroceras agreste,* and 848 and 450 μm in *Arion rufus.* As is obvious, in the eyes of the snails *C. nemoralis* and *T. hispida*, the shortening is only

5 to 9 μm, but in the eyes of the slugs it is *ca.* 400 (*A. rufus*) and 40 μm (*D. agreste*).

As we see here, the contribution of the first lens, the integument-cornea complex in the snails' eyes, is surprisingly small and in slugs, the refractive power of the integument-cornea complex looks very significant. However, at this point of our discussion, we still continue to argue that the lens, but not the integument-cornea, is the principal optical element of the eyes in terrestrial gastropods, just as in aquatic species. It is very likely that the optical properties of the lens in the terrestrial gastropods reflect the lens properties that their marine ancestors might have had.

The quality of the images produced by the isolated lenses of both terrestrial and aquatic gastropods demonstrates that the latter are corrected for spherical aberration (Gál *et al.* 2004). Therefore, we may conclude that lenses in both aquatic and terrestrial pulmonates, presumably possess a radial gradient of refractive index (*i.e.*, are optically inhomogeneous), irrespective of lens shape. However, to entirely eliminate spherical aberration it is not enough to have refractive-index gradients. The lens should be spherically symmetrical and have a ratio of focal length to lens radius of about 2.5 (Warrant and McIntyre 1993). Newell and Newell (1968) have shown that the lens in the terrestrial slug *Agriolimax reticulatus* has a focal length of 2.8 radii and, on that basis, concluded that the lens must have a graded refractive index.

The ideal spherical geometry, combined with a gradient of refractive index and an F-number of 2.9, endows *Lymnaea stagnalis* with a perfect aplanatic lens and superb optical image quality. Other species, like *Radix peregra*, *Physa fontinalis*, and *Planorbarius corneus* come close to the optimum, but imperfections in the optics such as low F-numbers (1.7, 1.8, 1.8, and 1.9, respectively) (Table 3) of the eyes manifest themselves as degradations in the quality of the image formed on the retina. Spherical aberration must be worse in the eyes of the terrestrial snails *Cepaea nemoralis* and *Trichia hispida* because of large, in relation to their focal length, apertures (0.7 and 0.8 compared to 0.3 in *L. stagnalis*) and low F-numbers (1.3 in *C. nemoralis* and 1.2 in *T. hispida*) as their eyes are designed to capture as much light as possible (Table 3) (Gál *et al.* 2004). The slug *Deroceras agreste* was found to have a high F-number equaling 2.5 and a spherical lens very likely free of aberration (Zieger *et al.* 2008).

Another source of lens imperfection, namely chromatic aberration, arises because the transparent material of the lens is invariably dispersive, that is, light of shorter wavelength is refracted by the material more strongly than light of longer wavelengths (Warrant and McIntyre 1993). However, chromatic aberration becomes important only when the diameter of the aperture exceeds about 500 μm (Land 1981). We therefore did not take chromatic aberration into consideration because the species studied have apertures in the range of 40 μm (*Ancylus fluviatilis*) (Meyer-Rochow and Bobkova 2001) to 140 μm (*Planorbarius corneus*) (Zhukov *et al.* 2002).

Even if the effects of spherical and chromatic aberration are negligible, the image produced by the eye can still be blurred. This limitation in image quality is referred to as diffraction.

In general, at any given wavelength, lenses with larger apertures will have narrower Airy discs (the central peak of a diffraction blur-circle), and thus suffer least from diffraction. For instance, aperture and Airy disc are 60 μm and 3.6 μm in *Lymnaea stagnalis*, 80 and 3.0 μm in *Deroceras agreste*, 92 and 1.5 μm in *Trichia hispida*, 107 and 1.6 μm in *Cepaea nemoralis*, and 140 and 2.0 μm in *Planorbarius corneus* (Tables 1 and 3). Larger apertures lead to losses in image quality due to aberration, and therefore for each eye design there must be some sort of compromise between diffraction and aberration.

Do the gastropods make use of their definitely advanced optical designs? It appears that even if eye optics can focus aberration free and diffraction-limited images, there are still anatomical limitations within the eye, which can destroy the potential for high resolving power of the gastropod eye.

Retinal geometry

If the position of the retina does not coincide with the sharp image plane (= position of the focal point within the microvillar layer), it would lead to wide receptive fields in individual receptors and thus result in considerable decreases of resolution. Such severely under-focused eyes occur in the terrestrial snails *Cepaea nemoralis* and *Trichia hispida*, where the sharp image falls just below the light-receiving microvilli within the retina (Gál *et al.* 2004). The underfocusing leads to a blurred image and loss of fine visual detail that the optics of these snails is able to provide.

The situation is even worse in the slugs *Arion rufus* and *Deroceras agreste* (Zieger *et al.* 2008) as well as the aquatic limpets *Latia neritoides* and *Ancylus fluviatilis* (Meyer-Rochow and Bobkova 2001) where the sharp image lies well outside the retina. Although refractive power of the integument-cornea-complex is very significant in slugs, such shortening of the focal length of the two lenses still does not even allow a blurred image to be formed within the retina in the eyes of *A. rufus* and *D. agreste*.

The optics of the eyes in these pulmonates cannot assist in focusing an image on their shallow retinae because to shorten the principal focal length of their lenses to place the focal point onto light-receiving layer would require a central refractive index of the lens of approximately 1.9 (Land 1981), which is well beyond that of even the densest crystalline proteins (Sweeney *et al.* 2007).

Such short offset of the lens and the retina seems to indicate that eyes in terrestrial snails and slugs as well as some aquatic limpets are not designed to receive a focused image and are likely to measure the average light intensity or quality over large angles rather than resolve fine image details.

Aquatic snails like *Lymnaea stagnalis*, *Radix peregra*, *Physa fontinalis*, and *Planorbarius corneus* are able to focus a sharp image on the light-receptive layer of the retina. It is due to the deepenings of the latter, resulting in the increase of the vitreous body volume (Bobkova *et al.* 2004a, Gál *et al.* 2004).

Finally, it seems that there is no definite demarcation between lens eyes that form an image and those that do not. Nevertheless, we argue that it is the inappropriate geometry of the retina and the size of the eye in general, but not the optics alone, that cause the biggest differences between the eye designs in the species examined here.

VISUALLY MEDIATED BEHAVIOR

Visual capabilities and behavior

Numerous generalizations with regard to vision in pulmonate gastropods were based on observations of *Cornu aspersum aspersum* or closely related terrestrial snail species (Wheeler 1921, Geismer 1935, Zanforlin 1976, Bailey 1981, Hamilton and Winter 1984). Not surprisingly, a common assessment was that pulmonates have poor vision, *i.e.*, that their eyes can detect general light levels and perhaps broad areas of light and dark regions but not much else. However, comparisons of the visual capacities of freshwater snails and limpets, through behavioral tests and estimates based on analyses of eye structure and optics (Vakoliuk and Zhukov 2000, Meyer-Rochow and Bobkova 2001, Zhukov *et al.* 2002, Vakoliuk 2005), with those of terrestrial snails and slugs (Hermann 1968, Zhukov and Baikova 2001, Bobkova *et al.* 2004a, Gál *et al.* 2004, Vakoliuk 2005, Zieger *et al.* 2008) suggest a considerable range of visual capabilities exists within the pulmonate lineage.

Gastropods exhibit two easily distinguishable kinds of visual behavior. First, they can use light to orient by moving either toward, or more commonly, away from regions of high light intensity, and these responses usually take the form of a phototaxis. Second, gastropods may respond to a sudden decrease in light intensity by withdrawing into their shell and adhering tightly to the substratum. The first behavior is clearly concerned with habitat selection, while the second is a defense against a predator that casts a shadow prior to attack (Land 1968).

In pulmonates the ocular system (= paired cephalic eyes), but no other sensory system, is involved in the phototactic behavioral response. This conclusion is the result of behavioral experiments with operated (eyeless) animals that did not show any sign of phototactic response, while intact molluscs did. Electrical recordings from the eye of *Lymnaea stagnalis* by Stoll and Bijlma (1973) gave only "on-responses", confirming that the shadow reflex was mediated by (1) dermal receptors, in *Nassarius reticulatus* (Linnaeus, 1758) according to Crisp (1972), (2) in the skin and along the mantle and in *L. stagnalis*, according to Stoll (1976), and (3) in the foot, lips, and tentacles as well.

The freshwater pulmonates *Lymnaea stagnalis* (Stoll 1972, 1973, 1976, Vakoliuk and Zhukov 2000) and *Planorbarius corneus* (Zhukov *et al.* 2002) clearly choose to move towards illuminated targets, displaying positive phototaxis. However, terrestrial pulmonate snails like *Cornu aspersum aspersum* (Wheeler 1921, Hamilton and Winter 1984), *Helix pomatia* (Geismer 1935), *Otala lactea* (Müller, 1774) (Hermann 1968), *Eupariphа (Helix) pisana* (Müller, 1774) (Zanforlin 1976), *Achatina fulica* (Zhukov and Baikova 2001), *Cepaea nemoralis*, and *Trichia hispida* (Vakoliuk 2005) as well as the slugs *Agriolimax reticulatus* (Newell and Newell 1968), *Arion rufus*, and *Deroceras agreste* (Zieger *et al.* 2008) approach the dark regions of an experimental arena and, thus, display negative phototaxis.

A negative phototactic behavior is fully compatible with the dim or even dark environments that terrestrial snails and slugs are most active in (Newell and Newell 1968, Rollo and Wellington 1981, Hamilton and Winter 1984, Hommay *et al.* 1998, Cook 2001, Vakoliuk 2005). In contrast, positive phototaxis is compatible with the preference for a well-lit environment as, for example, in *Lymnaea stagnalis* and *Radix peregra* that exhibit moderately amphibious lifestyles and live on the algal growth of well-illuminated surfaces at the water/land boundary (Purchon 1977, Vakoliuk 2005).

Counsilman *et al.* (1987) suggested that in the bioluminescent terrestrial snail *Dyakia striata* (actually *Quantula striata* (Gray, 1834): Haneda 1981) the light produced by this species may have a social function. Although intraspecific communication by light was rejected by Meyer-Rochow and Moore (1988) for the bioluminescent freshwater pulmonate limpet *Latia neritoides*, comparisons of the eyes between this limpet and the equally large, but non-luminescing species *Ancylus fluviatilis* showed that the light-producing *Latia neritoides* had a significantly larger eye, larger lens, and more extensive retina (Meyer-Rochow and Bobkova 2001).

First described by Hamilton and Winter (1982), an experimental procedure to determine response thresholds for black stripes of different widths and orientation was then modified by Zhukov and Baikova (2001) and Vakoliuk (2005). Zhukov and Baikova (2001) have been able to show that the terrestrial snail *Achatina fulica* discriminates vertical and horizontal black stripes as well as grating patterns of different frequencies. Discrimination tests revealed a strong preference for vertical bars over diagonal and horizontal bars of the same width in *Otala lactea* (Hermann 1968), a significant preference for horizontal bars over vertical ones in *Cepaea nemoralis* (Vakoliuk 2005), and no preference in *Lymnaea stagnalis, Planorbarius corneus* (Vakoliuk 2005), and *Cornu aspersum aspersum* (Hamilton and Winter 1984).

The experimentally determined resolution limits (Vakoliuk 2005) are well supported by our calculations of receptor spacings (for comparison in parentheses) (Gál *et al.* 2004) in *Lymnaea stagnalis, Planorbarnis corneus, Cepaea nemorais*, and *Trichia hispida*: 2.5°–5.7° (2.4°–5.2°), 1.4°–1.9° (1.4°–3.4°), 8.0° (8.0°), and >10.0° (13.0°). *Cornu aspersum aspersum* has a resolution limit of 14.5°-24.6° (Hamilton and Winter 1984) and *Otala lactea*, 0.9° for a single vertical bar and 2.4°–3.7° for a horizontal bar of similar dimensions (Hermann 1968).

However, we should keep in mind that behavioral response threshold estimates do not necessarily approximate actual sensory thresholds. Thus, our theoretically calculated performances of slug eyes show that a centrally-placed receptor subtends about 0.4° in *Arion rufus* and 4.7° in *Deroceras agreste*, so that acuity on purely anatomical grounds should be considered to be quite good in both species. Yet, behavioral tests show that these slugs do not respond until resolution thresholds of ca. 26° and ca. 90°, respectively, are reached (Zieger *et al.* 2008). The reasons for such disparity between anatomically-determined estimates and behaviorally-determined resolution limits are probably to be sought in features of either the visual processing or the optical system.

Our research has led us to conclude that anatomically the eyes of the two species of slugs investigated should be able to form images, but images that would lie in the wrong plane, well behind any retinal structures. The eyes of the slugs are designed to transmit spatially averaged intensity patterns of light and dark to the CNS for orientation and only one aspect of the visual environment, the overall pattern of light and dark, seems to be important for them. A sharp image is unnecessary and may even be a hindrance when the aim is to provide nothing more than orientation.

Newell and Newell (1968) estimated that two adjacent receptors subtend an angle of 15° at the center of the lens so that the visual acuity is poor in the eye of *Agriolimax reticulatus*. The authors presumed that the slug's eyes are adapted to detect changes in light intensity only and to operate at night.

Hamilton and Winter (1984) concluded that the poor vision of *Cornu aspersum aspersum* may correlate with primarily nocturnal habits. Geismer (1935) reported that in *Helix pomatia* a significant orientation response was released to a 24 × 20 cm black card of angular size extending at least 20° and positioned 25 cm away from the snail. According to Hermann (1968), another terrestrial snail, *Otala lactea*, can orient with respect to a target as small as 22.5°–30° at 25 cm distance. We have to conclude that the eye design in *Trichia hispida* and *Cepaea nemoralis* does not prevent image formation, but that resolution of the eyes can certainly not be great. Nevertheless, the eyes of these two snails are able to register not just the average light level but the quality of wide angles, and thus the crude direction, of a light source as well. This led us to suggest that the eye of terrestrial pulmonates, inherited from aquatic ancestors, has changed very little.

Secondarily aquatic pulmonates with higher visual needs are capable of image formation in both air and water. During their evolution, they have changed the terrestrial eye into a type that can function under water by deepening their retinae, so that the latter are capable of handling the increase in focal length required for vision under water (Bobkova *et al.* 2004a, Gál *et al.* 2004).

CONCLUSION

The terrestrial snails *Cepaea nemoralis, Otala lactea, Cornu aspersum aspersum, Achatina fulica*, and *Trichia hispida* are active under twilight conditions (*e.g.*, are crepuscular) and thus would need to be able to collect some light to be able to retain the image-forming ability of their eyes. Although their resolution is very poor, their eyes are able to register the average ambient light level as well as the quality of wide angles of light.

The slugs *Arion rufus* and *Deroceras agreste* are crepuscular but have high F-numbers of 8.5 and 2.5, respectively. It seems that the eyes of these gastropods have another visual task: they monitor environmental brightness and assist the animal in orientating towards dark places. The slugs simply do not need to perceive sharp images. The eyes of the limpets *Latia neritoides* and *Ancylus fluviatilis* also have no image-forming capacity, and the same conclusion as for the slugs may apply. However we do not know if the eyes of the limpets assist them in orienting towards dark, or toward light areas, and whether the somewhat larger eye of *L. neritoides* has something to do with the fact that this species can produce light when attacked (Meyer-Rochow and Moore 1988).

The eyes of *Lymnaea stagnalis* and *Radix peregra* are well adapted for vision in both watery and terrestrial habitats, and we suggest that the shape of the retina and the optics of the eyes of these snails match their amphibious life styles. The eyes of *Physa fontinalis* and *Planorbarius corneus* appear to have been less modified from those of their ancestors. Probably these snail species have lesser visual needs and depend more on other senses like chemo- and mechanoreception.

Pulmonate gastropods use their eyes primarily for the following two kinds of visual task: (1) discriminating objects and possible enemies in their environment and (2) monitoring the environmental brightness level to orient towards dark places. The first type of visual task is characteristic of the aquatic snails and is served by image-forming eyes; the second is typical of terrestrial snails and slugs and is best served by a blurred image.

The studies on pulmonates can provide a more solid basis than currently exists for generalizations on the sensory capabilities of the molluscs in Class Gastropoda. Aside from the morphological and functional ramifications, this research is important from an evolutionary and taxonomic standpoint. The eyes are used as identifying characters in taxonomy or as a basis for comparisons in systematic discussions. An underlying knowledge of the function of the eyes, and why they take on a particular shape or design will obviously enhance their use or disuse as taxonomic or phylogenetic characters. Finally, an understanding of the various eye designs can shed light on evolutionary relationships and serve as a springboard for future research.

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The genus *Buccinanops*: A model for eye loss in caenogastropods*

Andrés Averbuj and Pablo E. Penchaszadeh

Museo Argentino de Ciencias Naturales – CONICET and Facultad de Ciencias Exactas y Naturales – University of Buenos Aires
Avenida Ángel Gallardo 470, laboratory 57, C1405DJR, Buenos Aires, Argentina, andresbuj@macn.gov.ar

Abstract: The genus *Buccinanops* (d'Orbigny, 1841) (Caenogastropoda, Nassarriidae) is endemic to the SW Atlantic Ocean, and the name implies *no eyes,* due to the lack of visible eyes in adults. We recognize for the first time the occurrence of eyes during several developmental stages within *Buccinanops.* Eye spots in *Buccinanops cochlidium* (Dillwyn, 1817) were observed during intracapsular development and in hatchlings and juveniles. Eyes were histologically confirmed in embryonic cephalic tentacles; they were comprised of sensory cells, supportive cells, a lens, and an optic nerve cord. The ontogenetic history of the eyes of *B. cochlidium* is discussed.

Key words: Nassariidae, eye development, embryology, gastropod blindness, ultrastructure

Molluscs represent a group with a diversity of eye types and range of complexity of eye structure and are becoming of increasing interest when modeling evolution of eye development (Tomarev *et al.* 1997, Arendt 2003, Platcheski *et al.* 2005). In the caenogastropods, a pair of eyes is usually located on the outer side of the cephalic tentacles, embedded in the tentacle or on a small bulge at its base. Originally, a separate stalk contains the eye in the side of the head immediately posterior to the cephalic tentacle (*e.g.*, *Haliotis* Linnaeus, 1758) or partially separated as in the Trochacea (Hyman 1967, Fretter and Graham 1994).

The interest in eye development in gastropods includes topics such as the independent development of the eye in comparison with other classes of molluscs and eye regeneration (Gibson 1984, Bever and Borgens 2005). Even more interesting is the loss of eyes in eyed lineages. The loss of eyes has occurred several times in the gastropods. Within them, some non-related families are cited to have blind representatives such as the archaeogastropod *Pisulina* sp. Neville, 1869 (Neritiliidae) (Kano and Kase 2002), the neogastropods *Buccinanops* sp. (d'Orbigny, 1841) and *Bullia* sp. (Gray in Griffith and Pidgeon, 1834, da Silva and Brown 1985) (both Nassariidae), the opisthobranchs *Retusa* sp. Brown, 1827 (Retusidae) and *Cylichna* sp. Lovén, 1846 (Scaphandridae) (Mikkelsen 2002), and the pulmonates *Cecilioides* sp. de Férussac, 1814 (Ferussaciidae) (Heller *et al.* 1991). A unique and consistent explanation for eye reduction or loss is not agreed upon although it is generally associated with habitual burrowers or living in habitats where light does not reach such as caves or ocean abysses (Hyman 1967, Fretter and Graham 1994, Strickler *et al.* 2001, Kano and Kase 2002). Comparative studies among eyeless species or lineages could help our understanding of why eye reduction and loss occur in nature and complement the modeling of eye development.

The genus *Buccinanops* (Caenogastropoda, Nassarriidae) represents a group of seven species, all endemic to the SW Atlantic Ocean (Pastorino 1993, Rios 1994). The genus name means "*Buccinum without eyes*" due to the lack of visible eyes in the adults (d'Orbigny 1841). Within the genus, *Buccinanops cochlidium* (Dillwyn, 1817) is the largest species (Fig. 1A) and ranges from Rio de Janeiro, Brazil (23°S) to Patagonia, Argentina (42°S). Animals reach up to 110 mm in length and are gonochoristic.

A study of the intracapsular embryological development of *Buccinanops cochlidium* was recently conducted in the field and conditioned aquaria (Averbuj and Penchaszadeh, unpubl. ms). Females of the species attach the egg capsules to the callous region of their own shell. Between 1 and 20 embryos completed their development within the egg capsule after a period of 4 months and the ingestion of thousands of entire nurse eggs. The embryos hatch as 4 mm shelled, crawling juveniles.

Observations made during this study identified small dark spots at the base of the cephalic tentacles of the embryos of *Buccinanops cochlidium* while developing inside the egg capsules. These spots coincided with the description of eye location in the tentacle and aspect of pigmentation (Fretter and Graham 1994). The structure and location of those eyes is studied here. Although we tentatively identified these structures as eyes in the encapsulated embryos, the presence of these structures in juveniles is not confirmed, and they are lacking in the adults (Fig. 1A).

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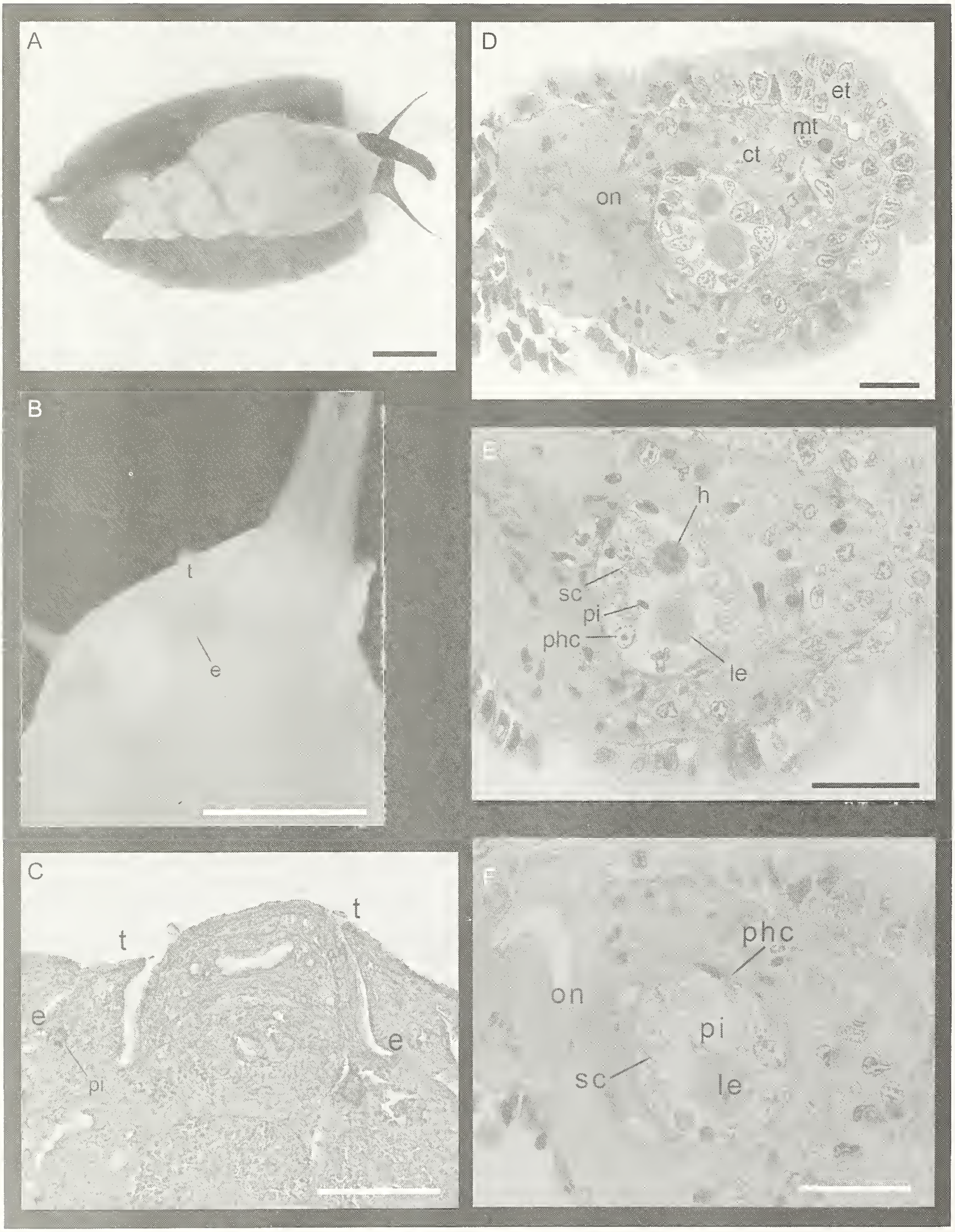
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MATERIALS AND METHODS

Samples were obtained from Villarino Beach, in San José Gulf, Argentina (42°25′S, 64°31′W). Collection was performed by scuba diving over muddy bottoms at depths varying between 5 and 15 meters. Females with attached egg capsules in different stages of development, juveniles, and adults were collected and taken to laboratory. The animals were maintained in seawater conditioned aquaria until processed. Salinity was fixed at 35 PSU and 12 °C, on a 12-12 h light: dark photoperiod. Individuals' shell length was measured with 0.1 mm precision vernier calipers.

Egg capsules representing all stages of embryonic development were detached from the shell of gravid females and dissected. Total shell length (TSL) of the embryos and free living individuals were measured at different stages of development (modified from Bigatti 2005) defined as: cell division, morulae, "veliger", late "veliger", coiling, pre-hatching, hatchling (all inside the capsules), juveniles, and adults. All measurements were made under a Zeiss stereoscopic microscope with a 0.1 mm precision ocular micrometer. Using a microscope, presence or absence of eyes was recorded at each of the different developmental stages as well as in hatchlings, juveniles, and adults. Whenever eyes were found, the cephalic tentacles containing the eye were dissected for histology studies. When the eyes were not visible, the whole tentacle was fixed and preserved for continuous sectioning.

Material was pre-fixed in 2.5% glutaraldehyde for two hours and rinsed in sodium cacodylate buffer, post-fixed with 2% Osmium for 1 hour, and rinsed again in sodium cacodylate buffer. Fixed specimens were serially dehydrated with ethanol in graded steps and embedded in Spur's epoxy resin. Sectioning of embedded specimens was done in 1 μm sections from the base of the tentacle to the apex. Slides were stained with Methylene Blue for optical microscopy.

When possible, sections of the eye were mounted on TEM copper grids and stained with 2% uranyl acetate (Reynolds 1963). This technique enabled the visualization of microvilli and/or cilia used to define cell types.

RESULTS

Eye spots were first recognized in all intracapsular embryos at a late "veliger" stage (2.9 ± 0.5 mm of total length; *N* = 39) when the cephalic tentacle is developed conspicuously and the foot and shell have already started to develop. Eyes were also observed at the pre-hatching stage (3.5 ± 0.44 mm; *N* = 38) and in hatchlings (4.0 ± 0.6 mm of total shell length; *N* = 626; Fig. 1B).

The eye is located at the basal region of the cephalic tentacles (Fig. 1B-C). In transverse section of the tentacle, the structure of the eye shows a basal membrane, a retina, and a lens (Fig. 1D-E). Two cellular types (probably photoreceptor and supportive cells) appear to be present and forming in the retina. One cell type shows condensed chromatin (euchromatin) which is observed as dark nuclei and corresponds to the photoreceptor cells. The supportive cells, which occur in a higher frequency than the photoreceptor cells, have less condensed chromatin (heterochromatin; Fig. 1F).

Pigmentation is present in both cell types in different degrees of density. The eye's maximum width in the embryos ranged between 35 and 40 μm (*N* = 8), in embryos measuring from 2 to 5 mm of total length. We could not identify an area for entrance of light to the eye in any section of the tentacles, as each eye was consistently surrounded by tentacle tissues (epidermal, muscular, and connective tissues; Fig. 1D).

The black spots were also recognized macroscopically in a single 15 mm crawling juvenile (total shell length), but we could not find an eye structure microscopically. Tentacles of an adult (60 mm of TSL) were also studied microscopically but no eye was found.

DISCUSSION

In this work we recognized for the first time the presence of eyes in the encapsulated late embryo and confirmed them histologically in late intracapsular embryo stages. Although there certainly are other species of snails without eyes in the adult individuals (in Argentina the genus *Olivancillaria* d'Orbigny, 1841 (Olividae) and other groups cited above) but where embryos probably have eyes, to our knowledge this is the first study on the embryonic eyes of a blind gastropod species. Loss or reduction of eyes is usually associated with living in poorly illuminated environments

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Figure 1. *Buccinanops cochlidium*. A, Adult specimen of *B. cochlidium* from Villarino Beach, Patagonia. B, Dark spots at the base of the tentacles of a pre-hatching embryo. C, Cross section of the cephalic region of a late "veliger" embryo. The eye on the left tentacle is pigmented. D, Cross section at the base of the tentacle. E, Detail of the eye with lens and humor and surrounded by tissues (connective, muscular, and epidermal). The optic nerve is shown. F, Detail of the eye at the base of the tentacle of a pre-hatching embryo, with a unique lens and different cell types. Abbreviations: e, eye; t, tentacle; on, optic nerve; le, lens; h, humor; pi, pigmentation; phc, photoreceptor cell; sc, supportive cell; ct, connective tissue; mt, muscular tissue; et, epidermal tissue. Scale bars = 2 cm (A), 1 mm (B), 100 μm (C), and 20 μm (D-F).

(Hyman 1967, Fretter and Graham 1994). In this case, *Buccinanops cochlidium* lives in shallow waters in a well-illuminated environment, yet individuals often are found to be buried a few centimeters in the muddy/sandy bottom. An exception to this observation is when the animals are feeding isolated or in groups on carrion.

Two cellular types appear to be present in the retina of *Buccinanops cochlidium*. Pigmentation is present in both types, but in aggregations of different densities. A lens, a basal membrane, and an optical nerve complete the structure of the photoreceptor organ. The lens sometimes appears as one big roundish structure, while in other cases it is smaller and accompanied by a second similar structure which is colored darker, resembling a humor (Fig. 1E).

Although a pair of dark spots was observed in a 15 mm (post-hatched) individual, it was not possible to confirm histologically the presence of eyes. Studying individuals in this size range would be important to determine whether the eye is conserved intact or modified, deeply embedded in the tentacle tissue, or if it degenerates as the animal ages. In snails measuring more than 20 mm of TSL, eye spots are not visible macroscopically; thus, the hypothesis that eyes degenerate in adults is a strong possibility. How does the degeneration occur? Kano and Kase (2002) discussed possibilities such as reduction in size, loss of retinal pigmentation, or sinking under the skin.

At the moment, TEM techniques are being used to complete ultrastructure information of the embryonic eyes and attempt to confirm the photoreceptor cell type, rhabdomeric or ciliary (Arendt 2003, Plachetzki *et al.* 2005). Tentacles of juvenile and adult individuals of increasing size ranges were preserved for later studies (continuous serial cut). If eyes occur in juveniles or adult snails, comparison of the ultrastructure with that of the embryo will be relevant in order to know whether it remains equal or if it is modified in type and number of retinal cells, as happens in other snails (Blumer 1996, 1998). We found no literature about modification or loss of embryonic eyes in species with no adult eyes. Additional work comparing the structure and function of *Buccinanops* eyes in related groups, such as the buccinid *Buccinum* sp. (Linnaeus, 1758) or the nassariid *Bullia* sp. (Brown 1982, Cernohorsky 1984, Allmon 1990) (with and without eyes, respectively) may also be insightful.

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Evolution of mollusc lens crystallins: Glutathione S-transferase/S-crystallins and aldehyde dehydrogenase/Ω-crystallins*

Joram Piatigorsky

Laboratory of Molecular and Developmental Biology, National Eye Institute, National Institutes of Health, 7 Memorial Drive, Building 7, Room 100, Bethesda, Maryland 20892-0704, U.S.A., joramp@nei.nih.gov

Abstract: Diverse crystallins (abundant water-soluble proteins) are responsible for the optical properties of transparent cellular eye lenses and are multifunctional proteins that have been recruited from stress proteins and enzymes by enhanced lens expression. The major (S-crystallins) and minor (Ω-crystallin) cephalopod crystallins were recruited from glutathione S-transferase (GST) and aldehyde dehydrogenase (ALDH), respectively. S-crystallins underwent multiple gene duplications while Ω-crystallin appears to be encoded in a single-copy gene. Except for one S-crystallin (considered a "molecular fossil"), S-crystallins lack enzyme activity due to mutation and insertion of a variable central peptide by exon shuffling. The Ω-crystallin is the sole crystallin in scallops. Scallop Ω-crystallin does not bind the co-factor NAD^+/NADH, lacks enzyme activity, and is a tetramer but migrates as a dimer by gel filtration, suggesting structural adaptations for crystallin function. Similar transcription factors (Pax6 among others) appear to drive high lens expression of crystallin genes in molluscs and other species consistent with convergent recruitment of the non-homologous crystallin genes.

Key words: cephalopods, scallops, enzymes, lens proteins, eye, gene expression

Cephalopods and scallops have camera-type, lens-containing eyes that appear grossly like the complex eyes of vertebrates. Indeed, the similarities in the overall structures and the common use of opsin family members for phototransduction within the photoreceptors have made the camera-type eyes of molluscs and vertebrates prototypical examples of convergent (independent) evolution of specialized organs (Packard 1972). However, studies showing that invertebrates and vertebrates employ similar transcription factors (especially Pax6) for eye development re-opened the idea that diverse eyes are monophyletic (derived once and are thus homologous due to common ancestry) rather than polyphyletic (derived independently multiple times) (Gehring and Ikeo 1999, Gehring 2004). Although the similarities in their transcriptional networks for eye development favored a single origin for eyes throughout the animal kingdom, it is not proof of monophyletic eye evolution for various reasons, including the facts that the eye developmental networks show differences among species, the networks are not used exclusively for one tissue or organ, and there are major developmental differences in eye development in different species, especially between invertebrates and vertebrates. In brief then, the existing data are generally consistent with a great deal of parallel evolution (independent recruitment of similar networks of genes) among the diverse eyes of different species.

Analysis of lens crystallins provides a detailed view of independent processes during eye evolution. Crystallins comprise 80-90% of the water-soluble proteins of transparent cellular lenses and are responsible for their optical properties (Bloemendal and de Jong 1991). The crystallins are diverse proteins that, despite their common function for lens refraction, often differ among taxonomic groups, *i.e.*, many crystallins are taxon-specific (Wistow and Piatigorsky 1988). In addition to being highly expressed in the lens, crystallins are often present in lower concentrations in other tissues where they serve non-refractive roles. Surprisingly, the taxon-specific crystallins are generally (not exclusively) identical or related to metabolic enzymes and consequently are called enzyme-crystallins (Wistow and Piatigorsky 1988, de Jong *et al.* 1989). The use of the identical protein for a refractive role in the lens and for one or more non-refractive roles in other tissues (as well as in the lens) has been called gene sharing (Piatigorsky *et al.* 1988) to convey the situation of having two or more distinct molecular functions directed by (*i.e.*, sharing) the identical protein-coding gene sequence (Piatigorsky and Wistow 1989, Piatigorsky 2007).

Many lens crystallins of invertebrates and vertebrates display gene sharing since they have non-refractive functions outside of the lens in addition to their refractive functions in the lens (Tomarev and Piatigorsky 1996). The present communication reviews the lens crystallins of the camera-type eyes of cephalopods and scallops. The existing data are consistent with these molluscan crystallins being recruited from the metabolic enzymes glutathione S-transferase (GST) and aldehyde dehydrogenase (ALDH). Many diverse proteins have been recruited to be crystallins in different species in vertebrates and invertebrates (Figs. 1 and 2).

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S-Crystallins

S-crystallins were named as such because they are the most abundant proteins in the squid lens (Siezen and Shaw 1982). S-crystallins are a large family of enzyme-crystallins that are homologous to the metabolic enzyme GST and are highly specialized for lens function (Tomarev and Zinovieva 1988, Tomarev *et al.* 1991, Tomarev *et al.* 1993, Chiou *et al.* 1995). They are differentially expressed in a radial gradient consistent with their expected role in focusing by creation of a refractive index gradient (Sweeney *et al.* 2007). The squid (Tomarev *et al.* 1992) and octopus (Tomarev *et al.* 1991) S-crystallin mRNAs are expressed strictly in the lens except that a few are also highly expressed in the squid cornea (Cuthbertson *et al.* 1992), consistent with the concept of corneal crystallins (Piatigorsky 1998, Jester *et al.* 1999, Piatigorsky 2001). The optical role of corneal crystallins, so named because of their abundance, remains elusive (Nees *et al.* 2002, Jester *et al.* 2005, Estey *et al.* 2007).

The cephalopod digestive gland expresses authentic GST that has high enzymatic activity; this active enzyme is expressed barely if at all in the lens (Harris *et al.* 1991, Tomarev *et al.* 1993, Tang *et al.* 1994). The three-dimensional structure of squid GST (known as GST σ) indicates that it has an unusually open active site that correlates with its high activity and has a characteristic dimer interface that differs from that of the vertebrate GST isoforms (Ji *et al.* 1995). By contrast with the digestive gland active GST σ, all but one (SL11 in squid; Lops4 in octopus) of the S-crystallins that have been examined lack enzyme activity (Tomarev *et al.* 1995).

The GST-related S-crystallin gene family (>20 members) was derived by gene duplications (Tomarev and Zinovieva 1988, Tomarev *et al.* 1992). The S-crystallins lost

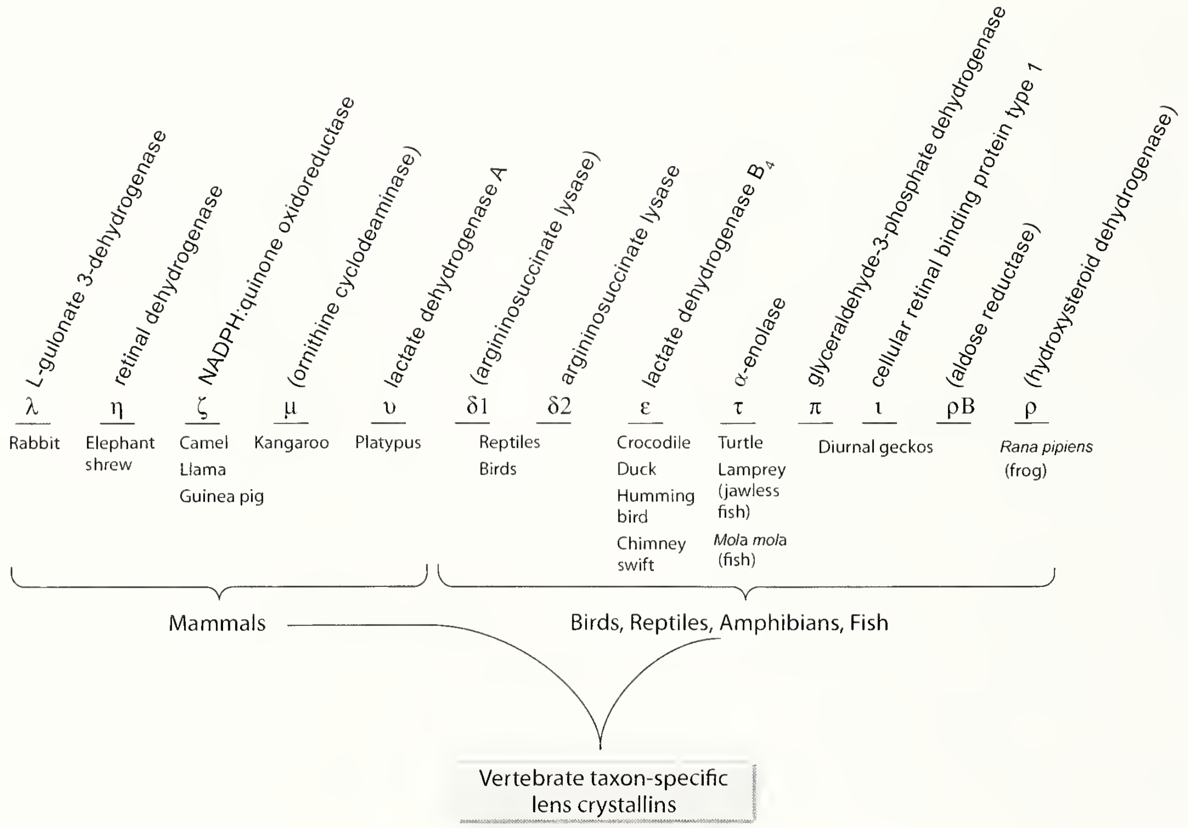

L-gulonate 3-dehydrogenase
λ
Rabbit
retinal dehydrogenase
η
Elephant shrew
NADPH:quinone oxidoreductase
ζ
Camel
Llama
Guinea pig
(ornithine cyclodeaminase)
μ
Kangaroo
lactate dehydrogenase A
υ
Platypus
(argininosuccinate lysase)
δ1
argininosuccinate lysase
δ2
Reptiles
Birds
lactate dehydrogenase B4
ε
Crocodile
Duck
Humming bird
Chimney swift
α-enolase
τ
Turtle
Lamprey (jawless fish)
Mola mola (fish)
glyceraldehyde-3-phosphate dehydrogenase
π
cellular retinal binding protein type 1
ι
Diurnal geckos
(aldose reductase)
ρB
(hydroxysteroid dehydrogenase)
ρ
Rana pipiens (frog)
Mammals
Birds, Reptiles, Amphibians, Fish
Vertebrate taxon-specific lens crystallins

Figure 1. Vertebrate taxon-specific lens crystallins. Enzymes in parenthesis lack activity. (Reprinted by permission of the publisher from *Gene Sharing and Evolution: The Diversity of Protein Functions* by Joram Piatigorsky, p. 62, Harvard University Press, Cambridge, Massachusetts, © 2007 by the President and Fellows of Harvard College.)

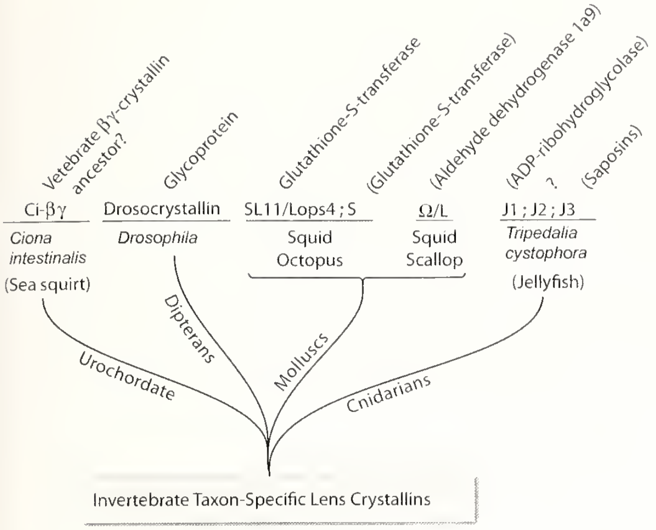


Figure 2. Invertebrate taxon-specific lens crystallins. Ci-βγ-crystallin in urochordates may be an ancestor of the vertebrate-βγ-crystallins (Shimeld *et al.* 2005, Piatigorsky 2006); however, *Ciona intestinalis* does not have a lens in its larval eye (there is no eye in the adult). Thus, the function of Ci-βγ-crystallin is not known. Enzymes in parenthesis lack activity. (Reprinted by permission of the publisher from *Gene Sharing and Evolution: The Diversity of Protein Functions* by Joram Piatigorsky, p. 64, Harvard University Press, Cambridge, Massachusetts. © 2007 by the President and Fellows of Harvard College.)

enzyme activity due to the insertion of an additional exon encoding peptides of variable length within the genes and to multiple point mutations sustained throughout the coding region of the genes. Only the squid SL11 gene and its orthologous gene in the octopus, Lops4, do not contain an additional central peptide acquired by exon insertion, as in the other S-crystallin genes; their encoded proteins are thus more closely related to the enzymatically active GSTσ than are the other S-crystallin proteins. Consequently SL11/Lops4 has residual GST activity (although much lower than GSTσ) despite being lens-specific. It is interesting, but speculative, to think of the SL11/Lops4 gene as a "molecular living fossil" of the enzyme-crystallin recruitment process during evolution, representing the original GST duplicate whose expression was directed to the cephalopod lens (Tomarev *et al.* 1995, Tomarev and Piatigorsky 1996).

Ω-Crystallin

Ω-crystallins of molluscs have been recruited from one or more members of the large group of aldehyde dehydrogenases (ALDHs) which metabolize a wide number of endogenous and exogenous aldehydes (Perozich *et al.* 1999, Vasiliou *et al.* 1999, Sophos and Vasiliou 2003). ALDHs are very ancient and found ubiquitously in microbes, plants, and animals. In eukaryotes alone, the ALDHs comprise at least 20 gene families. As a general rule, ALDHs with less than 40% identity in amino acid sequence are placed in different families and those with more than 60% identity belong to the same subfamily.

Due to the extensive number of ALDH genes, many showing high sequence identity and extensive gene conversions, their exact relationships to one another and time of gene duplications during evolution are uncertain. Ω-crystallin is a minor crystallin in octopus (ALDH1C1) and squid (ALDH1C2) lenses (Chiou 1988, Zinovieva *et al.* 1993) and is the only crystallin in the scallop lens (ALDH1A9), where it comprises ~70% of the water-soluble protein (Piatigorsky *et al.* 2000). It is also the sole crystallin protein present in the lens of the light organ of the squid *Euprymna scolopes* where it is known as L-crystallin (Montgomery and McFall-Ngai 1992). It is noteworthy that aldehyde dehydrogenase is the only enzyme known to have been recruited as a lens crystallin in both vertebrates and invertebrates and as a corneal crystallin in vertebrates (Tomarev and Piatigorsky 1996, Piatigorsky 1998). In vertebrates, the lenses of elephant shrews contain η-crystallin (ALDH1A8) (Graham *et al.* 1996), and ALDH3A1 and ALDH1A1 are the predominant corneal crystallins of mammals (Abedinia *et al.* 1990, Piatigorsky 1998, Jester *et al.* 2005). Although η-crystallin has retinaldehyde dehydrogenase activity (Graham *et al.* 1996), mollusc Ω-crystallins lack enzyme activity (Montgomery and McFall-Ngai 1992, Zinovieva *et al.* 1993, Piatigorsky *et al.* 2000). A mutation changing a critical cysteine to arginine in the active site of cephalopod Ω-crystallins has occurred, consistent with enzymatic inactivity of the encoded crystallin. Unexpectedly however, the active site sequence, including the critical cysteine, of the inactive scallop Ω-crystallin is intact. The reason for the absence of enzymatic activity of scallop Ω-crystallin appears to be due to the fact that it does not bind the required co-factor NAD^+ or NADH (Piatigorsky *et al.* 2000).

In contrast to the S-crystallin mRNAs in cephalopods, scallop Ω-crystallin mRNA is expressed weakly outside of the lens (gills, muscle) (Piatigorsky *et al.* 2000). Non-lens expression of Ω-crystallin is surprising because this abundant lens protein appears specialized for its non-enzymatic crystallin function. It remains possible that there are two closely related Ω-crystallin genes in the scallop, with the Ω-crystallin gene expressed solely in the lens and its duplicate expressed in other tissues. It is also possible that scallop Ω-crystallin will bind NAD^+ and has enzyme activity under different physiological conditions or can utilize an unknown substrate, allowing it to act as an enzyme under some circumstances. ALDHs are known to have wide substrate specificities as well as having non-enzymatic functions (Sophos and Vasiliou 2003, Estey *et al.* 2007). Thus, further investigations are necessary to reveal the possible enzymatic and/or other non-refractive function(s) of Ω-crystallin.

The squid and octopus Ω-crystallins have 78% amino acid sequence identity to scallop Ω-crystallin and 56-58% amino acid sequence identities to cytoplasmic ALDH1A1 and mitochondrial ALDH2 of vertebrates, while the scallop Ω-crystallin is 64% and 67% identical to human cytoplasmic ALDH1A1 and mitochondrial ALDH2, respectively (see Horwitz *et al.* 2006). Despite the close similarities of the cephalopod and scallop Ω-crystallins to both cytoplasmic ALDH1 and mitochondrial ALDH2 enzymes, phylogenetic tree analysis shows that the cephalopod Ω-crystallins cluster with the cytoplasmic ALDH1 proteins and the scallop Ω-crystallin clusters with the mitochondrial ALDH2 proteins (Horwitz *et al.* 2006).

Despite the sequence homology of scallop Ω-crystallin to ALDH1 and ALDH2, the native protein migrates as a homodimer (100 kD) by gel filtration chromatography rather than a homotetramer (200 kD) as expected for an ALDH1 or ALDH2 (Piatigorsky *et al.* 2000). Re-examination of scallop Ω-crystallin by on-line multi-angle laser light scattering showed, however, that Ω-crystallin is a tetrameric protein despite its chromatographic properties (Horwitz *et al.* 2006). It, thus, appears as if the anomalous gel filtration chromatography behavior of scallop Ω-crystallin reflects structural alterations, yet to be discovered, associated with its specialized crystallin function. In that connection, X-ray crystallographic studies of elephant shrew η-crystallin provided direct evidence for numerous structural features indicating that this recruited ALDH-crystallin displays characteristics of the cytoplasmic ALDH1 as well as of mitochondrial ALDH2 (Bateman *et al.* 2003). Mollusc Ω-crystallins await crystallographic studies.

Finally, mollusc Ω-crystallins lack an N-terminal leader sequence directing the protein to mitochondria (Piatigorsky *et al.* 2000, Horwitz *et al.* 2006). The absence of an N-terminal leader sequence is unexpected for scallop Ω-crystallin because, in contrast to squid and octopus Ω-crystallins, the phylogenetic tree analysis suggests that it was recruited from a mitochondrial ALDH2-like enzyme. All known mitochondrial ALDH proteins have a mitochondrial leader sequence, unlike the cytoplasmic ALDH1 proteins that do not (Piatigorsky *et al.* 2000, Horwitz *et al.* 2006). The absence of a mitochondrial leader peptide allows Ω-crystallins to fill the cytoplasm and serve refractive crystallin roles rather than be shunted into the mitochondria. Similarly, η-crystallin of elephant shrews lacks a mitochondrial signal sequence and is a cytoplasmic, ALDH1 protein (Graham *et al.* 1996). Cephalopod and scallop Ω-crystallins also have numerous sequence differences whose biological significances remain to be elucidated, as discussed elsewhere (Horwitz *et al.* 2006).

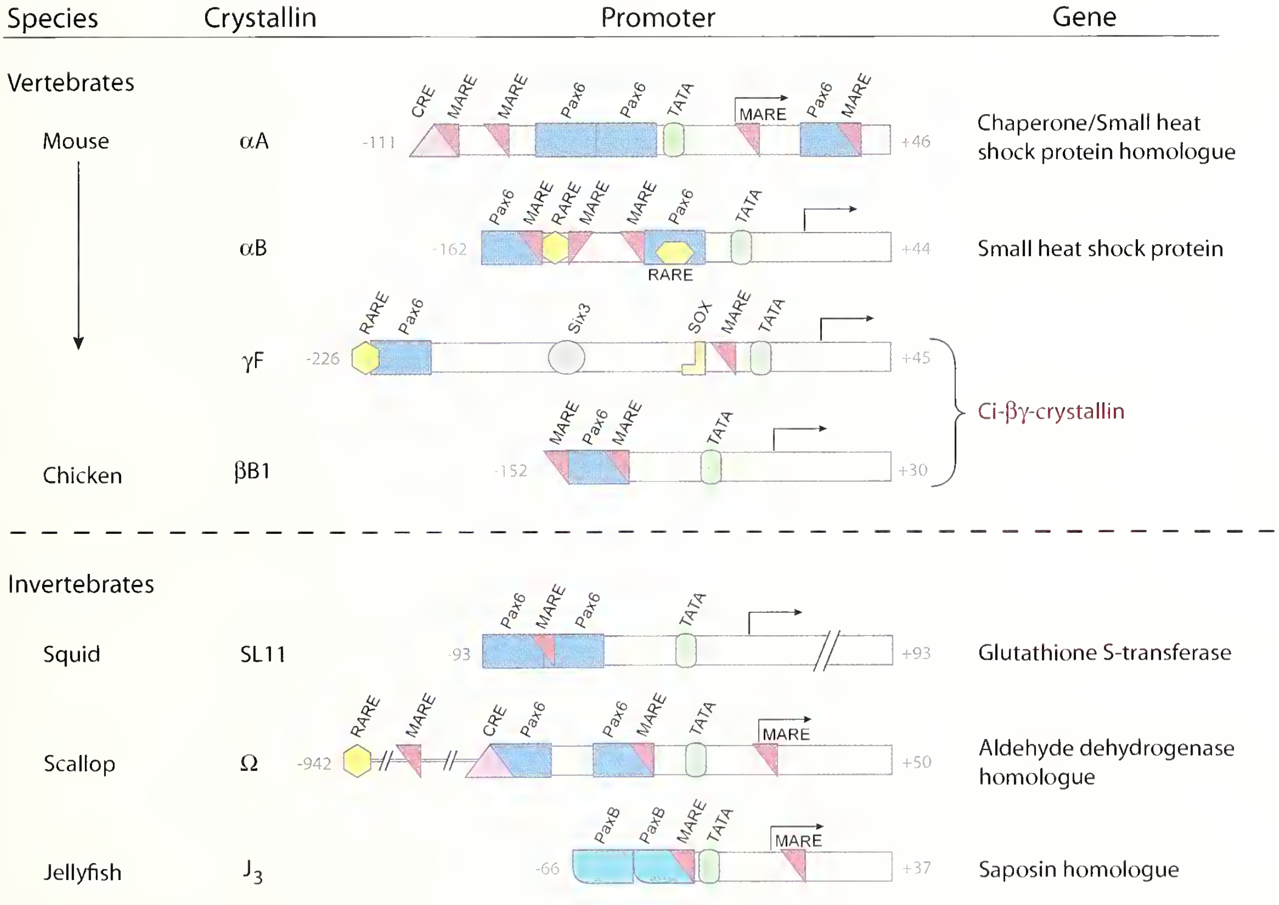
Crystallin gene expression

The hallmark of a water-soluble protein being a crystallin is abundance in the transparent lens. It has been suggested that 5% of the water-soluble protein of the lens be considered a minimum concentration for a crystallin (de Jong *et al.* 1994). Crystallin genes have complex patterns for expression in numerous tissues as well as being specialized for high lens expression consistent with their having been recruited from proteins with non-crystallin roles presumably by undergoing a period of gene sharing during evolution. Moreover, crystallin gene expression in the lens is exquisitely regulated to create a smooth gradient of protein concentration that is highest in the center of the lens and lowest at the periphery. Cephalopod crystallins amount to as much as 70% of the protein in the center of the hard lenses. Scallop crystallins have a much lower concentration as judged by the softness of the scallop lenses (personal experience). The gradient of protein concentration in the lens produces a corresponding gradient of refractive index allowing a focused image of incident light upon the retinal photoreceptors. In addition, the crystallin concentrations within the individual lens cells must be regulated to minimize refractive index fluctuations so that the cells are transparent (Benedek 1971, Bettelheim and Siew 1983, Delaye and Tardieu 1983).

Little is known about crystallin gene regulation in cephalopods or scallops; therefore, it is useful to apply our knowledge about the well studied mechanisms of crystallin gene regulation in vertebrates. Investigations on vertebrates have found that lens-specific expression of the diverse, non-homologous genes is regulated by a similar set of transcription factors that are involved in lens development (Cvekl and Piatigorsky 1996, Duncan *et al.* 2004). Foremost among them is Pax6, which plays a key role in lens development of all vertebrate lenses (Gehring and Ikeo 1999, Gehring 2005). Additional factors include members of the Maf family, Sox2 and retinoic acid receptors, among others (Duncan *et al.* 2004, Piatigorsky 2007). Regulation of lens preferred expression of non-related crystallin genes by similar transcription factors implies convergent evolution of crystallin gene regulation. In short, the data indicate that, in vertebrates, the recruitment of diverse proteins (often with stress protective or enzymatic functions) to become lens crystallins occurred by independent modifications of their gene regulatory motifs (*cis*-control elements) making them accessible to a restricted set of transcription factors that direct lens development.

Although scant, the available data indicate that crystallin recruitment in invertebrates has also occurred by independent convergent changes in gene regulation. Both S-crystallin and Ω-crystallin promoters have independently acquired DNA motifs that bind transcription factors from the same family that are used for vertebrate crystallin gene expression. A diagrammatic comparison of transcription factor binding sites in vertebrate and invertebrate crystallin promoters is shown in Fig. 3. Note that similar transcription factors (color-coded) bind to or activate the promoters of the non-homologous crystallin genes. Of particular interest are the Pax6 (PaxB in jellyfish) and Maf (MARE) binding sites on the various promoters since these transcription factors are critical for lens development. Functional studies showed that minimal squid promoter fragments for the SL20-1 crystallin gene and the SL11 gene are active in transfected rabbit lens epithelial cells (Tomarev *et al.* 1992). Promoter activity of these fragments depends upon AP-1 sites. AP-1 sites are also important for lens activity of vertebrate crystallin promoters (Piatigorsky and Zelenka 1992). The AP-1 sites in these squid crystallin promoters overlap with sequences similar to antioxidant and xenobiotic responsive elements which are involved in rat GST gene and guinea pig ξ-crystallin gene expression (Tomarev *et al.* 1992, 1994). GST and ξ-crystallin (which has quinine reductase activity) have physiological stress protective functions consistent with their genes being regulated by stress response elements and with the stress protective nature of vertebrate crystallin genes (de Jong *et al.* 1989, Horwitz 1992). The putative control elements of the S-crystallin promoters resemble the PL-1 and PL-2 (polyomavirus-like enhancer element 1 and polyomavirus-like enhancer element 2, respectively) *cis*-control elements of the lens-specific chicken βB1-crystallin promoter (Roth *et al.* 1991).

Although Pax6 appears necessary for establishing the eye field in cephalopods, it is uncertain at the present time whether or not it controls S-crystallin gene expression in the


Species
Crystallin
Promoter
Gene
Vertebrates
Mouse
αA
-111
CRE
MARE
MARE
Pax6
Pax6
TATA
MARE
Pax6
MARE
+46
Chaperone/Small heat shock protein homologue
αB
-162
Pax6
MARE
RARE
MARE
MARE
Pax6
TATA
RARE
+44
Small heat shock protein
γF
-226
RARE
Pax6
Six3
SOX
MARE
TATA
+45
Ci-βγ-crystallin
Chicken
βB1
-152
MARE
Pax6
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+30
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SL11
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TATA
+93
Glutathione S-transferase
Scallop
Ω
-942
RARE
MARE
CRE
Pax6
Pax6
MARE
TATA
MARE
+50
Aldehyde dehydrogenase homologue
Jellyfish
J3
-66
PaxB
PaxB
MARE
TATA
MARE
+37
Saposin homologue

Figure 3. Diagrammatic representation of *cis*-control elements in the promoters of crystallin genes of vertebrates (upper) and invertebrates (lower). Note the similarity in control motifs in the non-homologous crystallin genes. CRE, cyclic AMP-responsive element; MARE, Maf regulatory element; RARE, retinoic acid receptor regulatory element. Jellyfish PaxB has a Pax2-like paired domain and a Pax6-like homeodomain. Ci-βγ-crystallin is a urochordate protein and may be ancestral to the vertebrate βγ-crystallins (Shimeld *et al.* 2005, Piatigorsky 2006). (Reprinted from Piatigorsky (2006) with permission from Nature Publishing Group.)

lens (Tomarev *et al.* 1997). A functional Pax6, as judged by its ability to induce ectopic eyes in *Drosophila*, is expressed in the anterior segment of the squid lens, consistent with it having a role in S-crystallin gene expression. However, neither the posterior lens cells nor the embryonic squid lentigenic cells express Pax6 (Tomarev *et al.* 1997), where S-crystallin synthesis is active (West *et al.* 1994). Nonetheless, potential Pax6 binding sites have been identified in the SL20-1 and SL-11 squid crystallin promoters [A. Cvekl (pers. comm.) in (Tomarev *et al.* 1995)].

Experiments with the scallop Ω-crystallin promoter extend the evidence that lens-preferred crystallin gene expression in molluscs is controlled by transcription factors that resemble those regulating lens-preferred crystallin gene expression in vertebrates (Carosa *et al.* 2002). The scallop Ω-crystallin promoter sequence contains an abundance of motifs that are similar to those in vertebrate crystallin promoters. These include *cis*-elements for oxidative stress response factors (CREB/Jun; AP-1) and developmental transcription factors (Pax6; Maf) as well as other gene regulatory proteins. Moreover, the results of co-transfection and site-specific mutagenesis experiments are consistent with overlapping CREB/Jun and Pax6 sites in the Ω-crystallin promoter being functional (Carosa *et al.* 2002). Thus, the "lens nature" of the scallop Ω-crystallin promoter is completely different from that of other ALDH promoters, which is striking in view of the fact that the scallop Ω-crystallin gene is homologous to and shares high sequence identity with ALDH1 and ALDH2 proteins (Carosa *et al.* 2002, Horwitz *et al.* 2006). Taken together, the data indicate that stress response and eye developmental transcription factors regulate lens-specific expression of mollusc S- and Ω-crystallin genes as they do vertebrate crystallin genes.

Does Pax2 regulate scallop Ω-crystallin gene expression?

Cubozoan jellyfish, members of the ancient Cnidarians, are particularly interesting with respect to the well-established role of Pax6 in the evolution and development of eyes throughout animals (Piatigorsky and Kozmik 2004). Cubozoan jellyfish have camera-type, lens-containing eyes as do cephalopods, scallops and vertebrates. The complex eyes of the cubozoan *Tripedalia cystophora* are situated on four equally spaced rhopalia nestled in open cavities on the surface bell surrounding the jellyfish (Piatigorsky *et al.* 1989, Piatigorsky and Kozmik 2004). Each sensory rhopalium contains two of these lens-containing eyes, one larger and one smaller, situated at right angles to one another as well as four simpler eyespots comprising photoreceptors that lack lenses. The rhopalia also have a relatively large statocyst used for orientation. The complex eyes have a mono-layered cornea and transparent, cellular lenses which refract incident light without spherical aberration, indicating the presence of a gradient of refractive index similar to vertebrate lenses (Nilsson *et al.* 2005). Three distinct crystallins (J1-, J2-, and J3-crystallin) (Piatigorsky *et al.* 1989), with J1 comprising three extremely similar polypeptides (Piatigorsky *et al.* 1993), are responsible for the optical properties of the lenses of the *T. cystophora*.

Despite having sophisticated eyes, jellyfish and other investigated Cnidaria, lack Pax6 (Sun *et al.* 1997, 2001, Groger *et al.* 2000, Miller *et al.* 2000). Instead, Cnidaria (as well as sponges (Hoshiyama *et al.* 1998)) have PaxB, which has a Pax2/5/8-like DNA binding paired domain and a Pax6-like homeodomain. It appears then as if modern Pax2 and Pax6 genes evolved from a PaxB-like ancestor by duplication and diversification in higher metazoans (Piatigorsky and Kozmik 2004). Indeed, PaxB of *Tripedalia cystophora* is a functional as well as structural hybrid of Pax2/5/8 and Pax6. For example, PaxB has the ability to induce small ectopic eyes in *Drosophila* as does both Pax2 and Pax6 (Kozmik *et al.* 2003). With respect to crystallin gene expression, jellyfish PaxB activates the jellyfish crystallin promoters in co-transfection tests although its paired domain will not recognize a Pax6 DNA binding site (Kozmik *et al.* 2003). Pax2, but not Pax6, is also capable of low-level activation of the jellyfish J3-crystallin promoter, consistent with the presence of Pax2-like *cis*-control elements that do not recognize a Pax6 paired domain. Crystallin genes of other species are activated by Pax6 but not by Pax2 since their promoters have Pax6 but lack Pax2 binding sites.

It is possible, although not known at present, that the scallop Ω-crystallin promoter is responsive to Pax2 as well as to Pax6. As mentioned above, this promoter has two putative Pax6 binding sites (Carosa *et al.* 2002). Inspection of the sequences of these Pax6 binding motifs reveals that site 2 of the scallop Ω-crystallin promoter has a 3' C consistent with preferential binding by Pax2. Moreover, a Pax2 cDNA has been cloned from scallop eyes (Kozmik and Piatigorsky, unpubl. data). It remains to be shown that Pax2 is expressed in the scallop lens and is capable of activating the Ω-crystallin promoter. A Pax2 contribution to the regulation on Ω-crystallin gene expression in scallop lenses would be very interesting and be consistent with convergent evolution of crystallin gene recruitment in scallops and jellyfish.

SUMMARY

Cephalopods and scallops have complex camera-type eyes. Their transparent lenses have recruited crystallins from the widely distributed families of metabolic GST and ALDH enzymes. Crystallin recruitment occurred by independent mutations of the *cis*-control elements of their genes allowing their promoters to be activated by transcription factors used

for lens development. This is the same convergent evolutionary process that has been used in recruitment of the vertebrate crystallin genes. The GST-derived/S-crystallin genes of cephalopods are expressed specifically in the lens and have undergone many gene duplications, insertions and base changes. Except for one S-crystallin, the cephalopod S-crystallins are enzymatically inactive proteins.

The cephalopod and scallop ALDH-derived/Ω-crystallins are encoded in single-copy genes. They show high sequence similarity to vertebrate cytoplasmic ALDH1 and mitochondrial ALDH2 proteins. Phylogenetic tree analysis indicates (but does not prove) that cephalopod Ω-crystallin was recruited from a cytoplasmic ALDH1-like gene while the scallop Ω-crystallin was recruited from a mitochondrial ALDH2-like gene. If scallop Ω-crystallin was indeed recruited from a mitochondrial ALDH2-like protein, it has lost its mitochondrial leader to resemble a cytoplasmic ALDH1-like protein. Neither cephalopod nor scallop Ω-crystallins show enzymatic activity, however for different reasons. The cephalopod Ω-crystallins have a mutated active site; by contrast scallop Ω-crystallin has a wild type active site sequence but the protein is unable to bind the NAD or NADH cofactor required for activity. The scallop Ω-crystallin gene is highly expressed in the lens but, unlike the cephalopod S-crystallin genes, is also expressed to a lesser extent in tissues outside of the eye. Scallop Ω-crystallin appears to have made adaptive changes in conformation affecting some of its properties. Similar to the S-crystallin promoters, the Ω-crystallin promoters have undergone independent sequence modifications making them responsive to transcription factors used for lens development in vertebrates, consistent with convergent evolution of crystallin gene recruitment.

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Photoreception and the polyphyletic evolution of photoreceptors (with special reference to Mollusca)*

Luitfried von Salvini-Plawen

Zoologie, Universität Wien, A-1090 Wien (Vienna), Althanstrasze 14, Austria, Luitfried.Salvini-Plawen@univie.ac.at

Abstract: The general expression of the transcription factor gene *Pax-6* homologues and the overall presence of the photopigment opsin have cast doubt on the polyphyletic evolution of photoreceptors. Herein it is proposed that the evolutionary pathway of photoreceptors reflects two different, successive processes: (i) the (monophyletic?) differentiation of photoreception as such, mediated by a specific transcription factor gene (such as *Pax-6* or *sine oculis*) and (ii) the genetic information of that induction factor (normative unit for photoreception). The latter stimulates the (polyphyletic) differentiation of the photoreceptors themselves through its multiply convergent co-options with variable network-modifications (intercalation of different genes). The expression of transcription factor genes does not *per se* imply homology of the differentiated photoreceptors (but at most some pattern of homoiology). The differentiation of both receptor types, ciliary versus rhabdomeric, in one and the same cell during development (of veliger larvae; Blumer 1996) shows them to be interchangeable structures (mere morphs). Apparently dependent of functional requirements, the structural type of the receptive organelle has no direct bearing upon the homology identification of the photoreceptors. This obviates the need to propose separate (ciliary and rhabdomeric) precursor cells in metazoans. A possible primitive "dermal" receptive cell which was polyphyletically adapted for metazoan photoreceptors is discussed. The rich morphological diversity of photoreceptors (including the larval organs) in Mollusca appears to represent in-group differentiations. Their polyphyletic lines are surveyed and the fine structure of eyes of three pteriomorph Bivalvia species—*Lima lima* (Linnaeus, 1758) (with a subdivided retina), *Chlamys varia* (Linnaeus, 1758), and *Pseudamussium peslutre* (Linnaeus, 1771) (with incomplete proximal retina)—is reported.

Key words: eye evolution, morphogenesis of photoreceptors, photoreceptor structure, transcription factor gene *Pax-6*, *Lima* and *Pseudamussium* (Bivalvia)

Photoreceptors (photoreceptive cells) and more differentiated photoreceptive organs (ocelli, eyes) are crucial sensory equipment for environment information and for orientation and are, therefore, widely differentiated within the animal kingdom. Their morphologically diverse organization raises questions on evolutionary relationships. New knowledge provides new views, and these are discussed herein.

The pioneer in ultrastructure research on photoreceptors, R. Eakin (1963, 1965, 1968), advanced the hypothesis that the evolution of photoreceptors followed two phylogenetic lines: the first involved opsin-containing surface enlargements of the cilia or flagella membrane (ciliary type) and the second involved enlargements in the form of microvilli of the distal cell membrane (rhabdomeric type). Some photoreceptors, however, have cilia as well as microvilli (mixed type). In contrast, Salvini-Plawen and Mayr (1977) proposed a convergent, polyphyletic origin of photoreceptors in about 40-65 independent lines. Later, Salvini-Plawen (1982) refuted a restriction of the "rhabdomeric line" to the cerebral photoreceptors of Protostomia or Spiralia (Eakin 1979).

More recently, the widespread findings of the molecular prerequisites for photoreception have blurred the homology debate on photoreceptive organs with regard to monophyly versus polyphyly. Homology implies similar structures or characters that are based on the same singular (monophyletic) evolutionary origin. The debate refers, on the one hand, to the ubiquitous presence of the (homologous) photopigment opsin and, on the other hand, to the expression of transcription factor gene *paired-box 6* (*Pax-6*, Pax6) homologues and other genes in photoreceptive cells in Triploblastica. Molecular biologists rapidly postulated that all photoreceptive cells and organs (ocelli, eyes)—at least of Triploblastica—are monophyletically homologous (Gehring and Ikeo 1999, Gehring 2001, 2004, Arendt and Wittbrodt 2001, Arendt 2003). With respect to this new perspective, E. Mayr (2002: 226) concluded "The origin of eyes in 40 branches of the evolutionary tree was always considered to be an *independent* convergent development. Molecular biology has now shown that this is not *entirely* correct" [italics by the present author]. Subsequently Mayr explained (2002: 227) "When survival is favored by the acquisition of a new structure or other attribute, selection makes use of all available molecules already present in the genotype"—that also includes the multiple convergent co-option of a transcription factor gene for receptor cell differentiation (see below).

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MONOPHYLETIC PHOTORECEPTORS IN TRIPLOBLASTICA?

According to Arendt and Wittbrodt (2001: 1546), the polyphyly of photoreceptors would be restricted to Protista, Cnidaria, many "aberrant" ("ectoptic/extraocular/extraretinal") organs (pygidial eyes in Polychaeta, pallial photoreceptors in Bivalvia, or neural ocelli in Acrania/Cephalochordata) and the cerebral photoreceptors in "Bilateria". The monophyly of cerebral eyes in Triploblastica (excluding the bilateral Cnidaria and Ctenophora; cf. Salvini-Plawen 1978) is based upon the general presence of opsin and the shared expression of and regulation by the transcription factor gene *Pax-6* (Gehring and Ikeo 1999, Gehring 2001, 2004, Arendt and Wittbrodt 2001, Arendt 2003). It is, however, very difficult to comprehend such a homology in structurally very different organizations. Examples include complex eyes in Arthropoda, the inverse eyes of Vertebrata, or the everse eyes in Cephalopoda. Closer examination leads to a proposed subdivision of the evolutionary pathway of photoreceptors to reflect two different processes. The first is the (monophyletic?) differentiation of photoreception (later controlled by opsin photopigments) and mediation by a transcription factor gene that induces receptor cell differentiation. The second is the multiple stimulation by that induction factor to differentiate (polyphyletic) photoreceptors through multiply convergent co-options with variable network-modifications (intercalation of different genes).

In a strict sense, genetic expression leads to clear homology of morphological structures or organs only when (in the simplest case) they correspond by inheritance one-to-one to a genomic locus or its orthologues (in other species). More generally, however, genes interact with each other to effect a specific developmental function and thus form a hierarchical, developmentally interactive, regulatory network (Fernald 2000, Davidson and Erwin 2006). Transcription factor genes act fairly dispersed within such a network, and the *Pax-6* expression is not specific for photoreception. It is also involved in morphogenesis (structural differentiation) of the cerebral center as well as in chemosensory organs. In squids, besides in the developing eye (but not in the retina) and cerebral tissues, *Pax-6* additionally expresses in the arms (suckers) and in the mantle (Tomarev *et al.* 1997, Hartmann *et al.* 2003). Moreover, *Pax-6* generally functions in a wide range of processes in further cells, including the interactions with other proteins (*e.g.*, Simpson and Price 2002). As underlined by Wagner (2007) with respect to the transcription factors, the regulatory network within which *Pax-6* expression in *Drosophila* is embedded includes the genes *eyeless, eyes absent, dachshund*, and *sine oculis*—this differs from the detailed network in vertebrates as exemplified by *Xenopus* (Fig. 1). Thus, although they include *Pax-6* or a homologue, the gene regulatory networks for the development of the insect and vertebrate eyes are derived independently (by intercalation of different genes); in morphological terms, this reflects homoiology at most (representing convergent structures derived from a homologous character). Therefore, *Pax-6* homologues expressing within different regulatory networks do not infer photoreceptor homology (see also Zuckerkandl 1994, Fernald 2000, Nielsen and Martinez 2003). Moreover, the *sine oculis* gene of *Drosophila* (and *optix* of vertebrates) clearly existed at the very base of the metazoans (Pichaud and Desplan 2002, Stierwald *et al.* 2004). Not the *Pax-6*, but the *sine oculis* gene (or a homologue of it) might be the important ancestral transcription factor gene for receptor differentiation (see also Pineda *et al.* 2002). In any case, only gene regulatory networks that include an almost identical combination of transcription factors (called 'character identity networks' by Wagner 2007) appear to execute specific developmental programs that reflect homologous structures and organs.

There is a fundamental difference between potentially monophyletic, primitive photoreception of cells based upon a particular gene regulatory network, and polyphyletically differentiated, distinct photoreceptors or organs (ocelli, eyes) incorporating independently modified gene regulatory networks (which in Triploblastica includes *Pax-6* homologues). This discrepancy, underlined 30 years ago by Salvini-Plawen and Mayr (1977: figs. 2, 9) for dermal light sense versus photoreceptors, is often neglected (*e.g.*, Vanfleteren 1982, Gehring 2004). Such prerequisite primitive cells with a transcription factor gene for receptor differentiation possibly still have "dermal" light sensitivity (sensitive cells without pigment; cf. Millott 1968). This would be similar to the case in Echinodermata (Yoshida and Takasu 1984), particularly in the echinoid *Paracentrotus* (Czerny and Busslinger 1995, cf. also Arendt and Wittbrodt 2001). It would also correspond to the general sensitivity involved in the shadow reflex ("off response") in many molluscs and especially of the pallial lobes in Bivalvia (Kennedy 1960, Mpitsos 1973, Messenger 1991, Morton 2001). The particular gene regulatory network of light-sensitive dermal cells became—by selection—the functionally successful, normative pattern or induction unit for photoreception. It was then repeatedly co-opted to

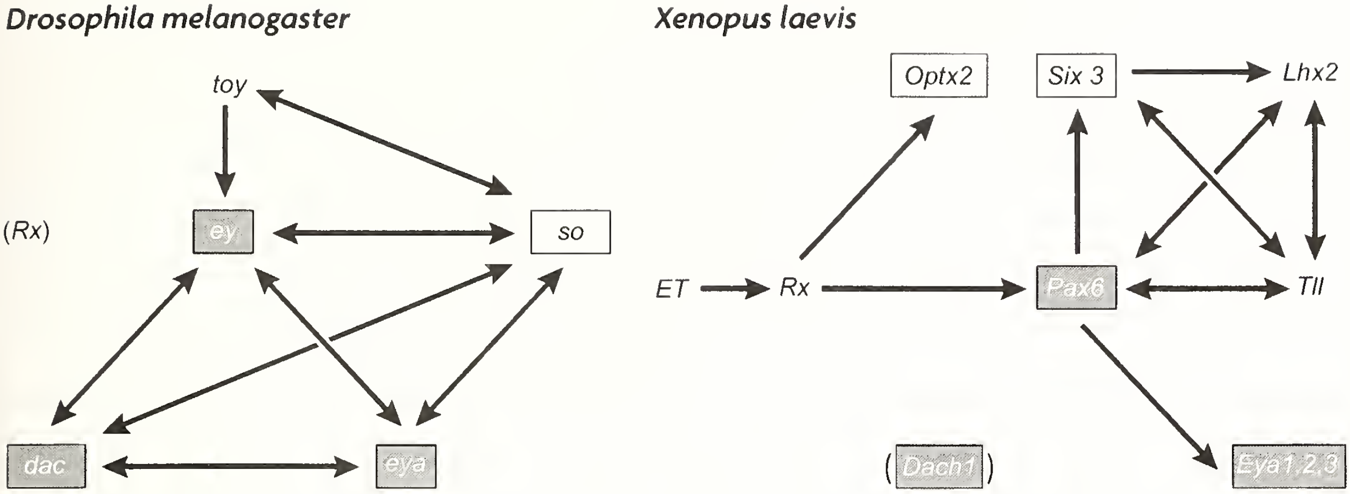

Figure 1. The non-homologous genetic regulatory networks for eye development in insects (*Drosophila*) and vertebrates (*Xenopus*); orthologous genes in grey boxes, paralogous genes in white boxes (after Wagner 2007). In *Drosophila*, the gene *twin of eyless* (toy) activates *eyeless* (ey) which is necessary for eye development; its network includes the genes *eyes absent* (eya), *dachshund* (dac), and another transcription factor *sine oculis* (so). In *Xenopus*, the gene *retinal homeobox* (Rx) is essential and regulates *paired-box 6* (Pax6, a homologue of *eyeless*) which includes in its network homologues of *eyes absent* (Eya1,2,3) as well as the *sine oculis homeobox* homologue 3 (Six 3) and its paralogue Optx2. A Rx homologue is also present in *Drosophila* but not involved in eye development. The non-homology of both networks is supported in vertebrates by Eya1,2,3 which do not regulate the *dachshund* homologue Dach1, as well as by additional genes involved.

serve as the foundation for the independent structural differentiation (morphogenesis) and evolution of photoreceptors. This initial normative induction unit, however, multiply modified its gene regulatory network by intercalation of different genes (see also Gehring and Ikeo 1999, Gehring 2001, 2002), yielding the variously differentiated, polyphyletic photoreceptors and organs (see Wagner 2007). The expression of certain specific factors such as *Pax-6* of the (otherwise not identical) network has been mistaken for homology of the photoreceptors themselves. As outlined elsewhere (Salvini-Plawen 1998a: 139), gene expressions appear to reflect normative regulators only and do not imply homology of the induced structures. Moreover, the hypothesis by Arendt and Wittbrodt (2001) of a homology of cerebral photoreceptors throughout all "major bilaterian branches" suffers from subjectivity: it claims discrepancies to be secondary losses and disregards more precise homology criteria.

Molecular biologists often are not necessarily aware of the synchronously differentiated organ systems within the complex "bauplans". First, the two nervous systems (including sense organs) of Triploblastica—gastroneural (at least in Spiralia) versus epineural—are not homologous and may simultaneously be present (Salvini-Plawen 2000). Second, it is functionally consistent that the photoreceptors of directedly moving organisms ("bilaterians") became differentiated by selection pressure (stimulation) at the anterior body and connected to the cerebral ganglia. This condition parallels the "pygidial" photoreceptors in several retrograde-moving Polychaeta-Sabellidae (Purschke *et al.* 2006) as well as the photoreceptors at the exposed mantle edge in Bivalvia (see below). Such conditions, however, reflect a general selection pressure towards convergent formation. Restricted solutions based on physical laws also play a role (Fernald 2006). Location *per se* is not proof for homology—unless other criteria also provide evidence. Such criteria include relationship, continuity, structural and/or developmental intermediates, and an identical gene regulatory network.

Thus, the present analysis focuses not on the origin of initial light sensitivity (which might be monophyletic) or the differentiation of photoreception as such. Rather it deals with the multiply stimulated "subsequent divergent, parallel and convergent evolution" of photoreceptors (Gehring 2004: 707 which in fact represents polyphyly). It involves independently modified and co-opted gene regulatory networks in which different genes are intercalated (see also Gehring and Ikeo 1999, Gehring 2001, 2002). This process leads to the convergent, selectively adapted, polyphyletic photoreceptor cells and organs (ocelli, eyes). Regulator genes are merely competent for the transcription towards photoreceptive cell differentiation but not for the adaptively convergent-stimulated, structural differentiation of the multiple cerebral and "ectoptic/extraocular/extraretinal" photoreceptors themselves.

PHOTORECEPTIVE MEMBRANE ENLARGEMENTS

With respect to the distribution of the two different types of photoreceptive membrane enlargements, a phylogenetically inherited occurrence can be attributed only to a taxon level ranging between phylum and order (Vanfleteren and Coomans 1976, Salvini-Plawen and Mayr 1977). This thoroughly contradicts the separation of photoreceptor evolution into a protostome (rhabdomeric) and a deuterostome (ciliary) line (Eakin 1963, 1965, 1968).

In addition to the ciliary and rhabdomeric receptor types, a third type called "diverticular" was recognized (Salvini-Plawen and Mayr 1977, Salvini-Plawen 1982). It originates from non-epidermal (sunken, ganglionic), aciliary cells (neurons) which have an unpleated receptor surface or show some diverticular membrane enlargements. An example is the ganglionic pigment-cup ocelli (Hesse cells) in the neural tube of *Branchiostoma*; they lack both cilia and microvilli. Such cells secondarily differentiated their cell membrane to represent surface-enlarging diverticulae. Without knowledge of their genesis, they can easily be confused with the rhabdomeric type (Salvini-Plawen 1982). Although later authors (Ruiz and Anadon 1991) claimed that this type was likewise rhabdomeric, *Pax-6* is not expressed in these Hesse cells (Glardon *et al.* 1998). Accordingly, dermal receptor cell differentiation by that induction factor (normative unit) is not involved. The seemingly exceptional condition in *Salpa*—with neurally derived, aciliary receptor cells with their hyperpolarizing "off-response" (Gorman *et al.* 1971; see below)—coincides with the diverticular receptor type (Salvini-Plawen 1982). This is also valid for the hyperpolarizing response of two photosensitive neurons of the abdominal ganglion in *Aplysia* Linnaeus, 1767 (Krauhs *et al.* 1979, Andresen and Brown 1982; see below). They also lack cilia and microvilli (diverticular type).

In view of such a condition, one of the pressing questions is why photoreception involves, on the one hand, ciliary and, on the other hand, microvillar (rhabdomeric, diverticular) membrane enlargements. Muñoz-Cuevas (1975) as well as Vanfleteren and Coomans (1976) presented an induction theory with a ciliary structure as an organizer (contradicted by Salvini-Plawen 1982 and Yamamoto 1985). Eakin (1979) advanced a concept based on mutations (discussed and contradicted by Salvini-Plawen 1982). Both hypotheses are inconsistent. In fact, most ciliary type receptors represent phylogenetically young organs (Vanfleteren and Coomans 1976, Salvini-Plawen and Mayr 1977, Burr 1984). Based on structural organization, ciliary reception has apparently changed to rhabdomeric reception in certain cases. These considerations point to ciliary differentiation as the initial (and/or original) type. This led Salvini-Plawen to propose a functional dependence and to conclude (1982: 151) that "the polyphyletic diversity of photoreceptors appears to be due to different functional requirements at different times during radiation of evolutionary lines."

Although functional correlations are still poorly understood, several investigations support such a principal dependence. Some earlier findings in Platyhelminthes-Polycladida (Ruppert 1978, Eakin and Brandenburger 1981, Lanfranchi *et al.* 1981) of ciliary receptors in larvae (adult photoreceptors are rhabdomeric) advanced the idea of potential structural change according to different functional requirements in larvae and adults.

In Gastropoda, the veliger larvae (but not others, see below) possess photoreceptors. They represent the organs of the adult organization precociously advanced into the larval stage (acceleration; Salvini-Plawen 1980b and below). The ocelli or eyes of adult gastropods typically show a rhabdomeric receptor structure (summarized in Blumer 1996); some species, however, have ciliated and microvillous receptors (mixed type; see below). Because of acceleration, a rhabdomeric receptor structure should also be present in the larval ocelli. This indeed is the case in short-term or actaeplanic veligers (Hughes 1969, Chia and Koss 1978), whose rhabdomeric photoreceptors might be sufficient for the short stay in the water column. Based on the argument that the photoreceptive demands in larvae with a long pelagic life (long-term or teleplanic larvae) will change accordingly, we might expect the receptive structure to change. Besides the evidence in Platyhelminthes-Polycladida (above), the finding of ciliary photoreceptive structures in the long-term veliger larva of *Ilyanassa obsoleta* (Say, 1822) (Crowther and Bonar 1980) supported this point of view.

In accordance with this hypothesis (Salvini-Plawen 1982, 1988), the photoreceptive structures in long-term (teleplanic) veligers were then demonstrated to correlate with the ciliary type (Blumer 1994, 1995, 1998, 1999). Moreover, the investigations by Dilly (1969) on *Pterotrachea mutica* (Lesueur and Peron, 1817), and by Blumer (1999) on *Atlanta peroni* Lesueur, 1817, revealed that a permanent pelagic life without a benthic phase (such as in Gastropoda-Heteropoda) might also be correlated with ciliary photoreceptors, even if variously adapted. Other heteropods, such as *Carinaria* Lamarck, 1801, however, still differentiate close to metamorphosis a photoreceptive segment of presumed rhabdomeric type. Structurally, this represents a mixed type (Blumer 1998) with a dual function. Thus, short-term (actaeplanic) larvae that spend less than six weeks in the plankton (Scheltema 1989) are correlated with rhabdomeric receptors whereas long-term pelagic life (larvae/adults) functionally correlates with ciliary receptors. Finally, Blumer (1996) demonstrated for teleplanic veliger larvae that the photoreceptive part of sensory cells themselves is successively altered during the life cycle: microvilli (for rhabdomeric structure) as well as photoreceptive cilia (monociliary to polyciliary) are added.

These conditions support the idea (Salvini-Plawen 1982) that the ciliary photoreceptive structure is adaptive for a pelagic existence, *i.e.*, is due to the functional requirements for this environment. This may refer to the reception of different wavelengths or to the sensitivity. Mobile benthic life no doubt demands that photoreceptive organs sense for orientation and/or vision (correlated to rhabdomeric structure?). In contrast, larvae within the water column need information on the direction of the light (correlated to ciliary structure?) for optimal feeding close to the surface and thus for the diurnal migration to that layer (periodicity). In sessile or semi-sessile animals, photoreceptors must recognize light/shadow (antipredatory) conditions with a hyperpolarizing "off-response" of (ciliary) receptor potential (cf. also "dermal" light sensitivity). Accordingly, this is apparently correlated with detecting changes in light intensity (decrease or light/shadow reactions) in very slow moving or sessile animals, with color changes including dorsal/ventral reactions and paling response in pelagic organisms, and with rhythmic behavior like diurnal migration of plankton and/or circadian rhythms (Land 1968, 1984, Salvini-Plawen 1982, 1988). In vertebrates, this appears to be performed by the dorsal (pineal and parietal) eyes, in direct inheritance of the (originally paired) ocellus in larvae of Tunicata-Ascidiacea (Hamasaki and Eder 1977, Salvini-Plawen and Mayr 1977). A long-term filter-feeding nourishment—during which the perception of light direction was sufficient—may explain why the ciliary receptor type was genetically firmly anchored. Then, when the shift to a directed food uptake stimulated the differentiation of new, repetitively (iteratively) homologous lateral eyes, this receptor type was retained (*i.e.*, not changed to the rhabdomeric morph). Rhabdomeric photoreceptive organs, however, may be called upon to correlate yes/no registrations of light stimuli with a depolarizing "on-response" (existence and/or increase of light intensity and direction); this would be more favorable for orientation.

HOMOLOGY AND THE PRECURSOR CELL STRUCTURE

Results with veliger larvae (Blumer 1996) show that one and the same photoreceptive epidermal cell can change its receptive surface structure according to function. This implies that photoreceptive structure has no direct bearing upon the identification of homology of the photoreceptive organs. A case in point is the Gastropoda. The same also holds true for Placophora, in which the monophyletic aesthetes and shell-eyes are ciliary and rhabdomeric structures. These structures are interchangeable and thus merely functionally conditioned different morphs. In other animal groups, however, the conditions appear to be different. In Sipunculida, for example, the inverse cerebral ocelli in the teleplanic larvae are not continuous with the adult everse cerebral tubes (Salvini-Plawen and Mayr 1977); the larval ocelli possess rhabdomeric receptive cells and appear to be homologous to the inverse pigment cup ocelli with rhabdomeric structure in polychaete larvae (Blumer 1997; not considered by Arendt and Wittbrodt 2001). Similarly, many members of Polychaeta have different organs for photoreception during their life cycle (Purschke *et al.* 2006): the larval ocelli may be retained as adult cerebral photoreceptors but more usually are replaced by adult cerebral organs (*e.g.*, Eakin and Westfall 1964, Rhode 1993). In late larvae, therefore, both pairs of photoreceptive organs may be visible (*e.g.*, in nectochaeta larvae of *Polydora* sp. (Spionidae) with outer larval and inner adult ocelli; pers. obs.). Only some of the pigmented or unpigmented larval ocelli investigated so far possess ciliary receptors. The altered photoreception requirements of larval and adult polychaetes, however, are reflected in the usual discontinuous differentiation of separate, non-homologous organs (versus/in contrast to Bartolomaeus 1992). The general predominance of rhabdomeric structure points to well-established adaptation (directed movement: cerebral photoreceptors) and, hence, to strong genetic anchorage. In contrast, ciliar structure appears to be the initial adaptive answer to photoreceptive requirements (*e.g.*, light-shadow reaction), with the rhabdomeric structure as a subsequent differentiation. The ciliary type is, thus, more frequently (still) represented in phylogenetically relatively young organs (Vanfleteren and Coomans 1976, Salvini-Plawen and Mayr 1977, Burr 1984). Such "new" requirements are evident in the teleplanic veliger larvae versus the "old" photoreceptors in adult gastropods.

This casts a new perspective on the homology criteria as related to photoreceptors. The results from long-term veligers considerably decrease the value of "compositional correspondence" of Remane's (1952) three principal homology criteria (positional correspondence, compositional correspondence, structural, and/or developmental intermediates). The ability to replace the receptive structure deletes one prominent morphological character for detailed homology comparison. Moreover, "positional correspondence" is also of limited value, as outlined above for cerebral photoreceptors. This diminishes the possibility to recognize convergences or parallelisms, unless there is different innervation or gene expression. At the same time, this contradicts the arguments of Arendt and Wittbrodt (2001: figs. 8(c), 9) and Arendt (2003) for separate precursor cells (ciliary versus rhabdomeric with opsin orthologues) in "Bilateria": one and the same cell can alter its photoreceptive part from a ciliary to a rhabdomeric type (Blumer 1996). Thus, the precursor cells (*e.g.*, "dermal" light sensibility) probably corresponded to a neutral, "mixed" or combined type with cilium and microvilli (Arendt and Wittbrodt 2001: fig. 8(b)). This condition was already proposed by Salvini-Plawen and Mayr (1977: figs. 2, 9) and is supported by recapitulation in cephalopods (Yamamoto 1985).

Gehring's (2001, 2004) two-cell model of a sensory and a pigment cell as the original condition in metazoans is no longer realistic: two types of sensory cells are present. The first are so-called phaosome photoreceptors, which are single cells with an invaginated vacuole housing the receptive organelles (*e.g.*, Clément and Wurdak 1984, Purschke 2003) which generally show no shading pigment. The second are receptor cells with intracellular pigment. Although verified in only in a few photoreceptors, a sensory cell with cilium + microvilli + intracellular pigment probably represents an early photoreceptive cell associated with shading pigment. In gastropods, pigmented sensory cells are differentiated in conservative groups, such as in *Patella* Linnaeus, 1758 (Docoglossa; Hesse 1902, Marshall and Hodgson 1990) and in *Haliotis* Linnaeus, 1758 (Vetigastropoda; Hesse 1902, Tonosaki 1967, Kataoka and Yamamoto 1981). They are also present in several other representatives (*e.g.*, *Ilyanassa* Stimpson 1865, *Murex* Linnaeus, 1758, *Aplysia*, *Pleurobranchus* Cuvier, 1804; Hesse 1902, Hughes 1969, Gibson 1984). Photoreceptive cells with pigment granules are likewise present in the veliger larvae of *Smaragdia* Issel, 1869 and *Strombus* Linnaeus, 1758, in *Octopus* Cuvier, 1798, and other cephalopods as well as in additional animal groups (Eakin 1972, Yamamoto 1985, Blumer 1995).

Going further back in evolution, the Cnidaria clearly point to a single cell type with a mixed/combined or a ciliary receptor (with opsin) as being the primary precursor structure for photoreception. Although they lack a cerebral center, Cnidaria possess an astonishing range of various "ectoptic/extraocular/ extraretinal" photoreceptors (Salvini-Plawen and Mayr 1977), albeit without *Pax-6* expression (Sun *et al.* 2001). They are generally of the ciliary type, though microvilli may also be present (*e.g.*, Singla 1974, Singla and Weber 1982, Blumer *et al.* 1995, Nordström *et al.* 2003). Accordingly, the type of opsins appears to be more closely related to that of the ciliary receptors or c-opsins (Plachetzki *et al.* 2007, Suga *et al.* 2008). In *Leuckartiara* (Anthomedusae) the sensory cells with microvilli, combined with an unmodified cilium (Singla 1974), correspond fairly well to the precursor cells (*e.g.*, "dermal" light sensibility) proposed by Salvini-Plawen and Mayr (1977: figs. 2, 9). Also, the receptor cells in the scyphozoan *Cassiopea* as well as in the cubozoans *Tamoya* and *Tripedalia* are pigmented (Bouillon and Nielsen 1974, Yamasu and Yoshida 1976, Laska and Hündgen 1982). All these presumed structural prerequisites (cilium, microvilli, and pigment granules) may also be combined within one cell. An example is the photoreceptive cell of cubozoan planula larvae (Nordström *et al.* 2003). It has no neural connection to any other cells and is structurally intermediate between a photosensitive dermal cell and a primitive organ.

The presumed light-sensitive cells in certain sponge larvae (parenchymulae of *Amphimedon queenslandica*, formerly *Reneira*; Porifera-Haplosclerida) each possess a long cilium and are pigmented. Though neither neurons nor opsins are present (Plachetzki *et al.* 2007, Suga *et al.* 2008), they react to changes in light intensity (Leys and Degnan 2001).

Thus, the primitive condition is reflected by a single precursor cell type (also Arendt *et al.* 2004). As already proposed (Salvini-Plawen and Mayr 1977), cells with a cilium and with some microvilli (mixed type) probably represent the basic type for (dermal) photoreception in Metazoa. This basic type is then convergent-adaptively modified for morphogenesis. Opsins are lacking in poriferans. This supports the morphological classification of the sponges as Parazoa as opposed to the Histozoa or Gastrozoa (Salvini-Plawen 1978, 1998b). It also reflects the presence of non-opsin controlled ("dermal") photoreception in Metazoa. Accordingly, photoreception evolved independently of and prior to the differentiation of opsin photopigment. Later, an opsin-like protein arose as a visual pigment in basal Histozoa/Gastrozoa (see also Plachetzki *et al.* 2007). Due to functional demands/adaptive "needs", opsin paralogues (by gene duplication) were differentiated within the same cells (see Blumer 1994). A correspondingly adapted opsin type became incorporated into the membranes of cilia. In the case of mixed receptors, a paralogue with a different type of transduction cascades additionally became incorporated into the microvilli (see also Arendt *et al.* 2004, Fernald 2006). This implies that two or more opsin paralogues can be differentiated within the receptive cells and would explain the expression of the "wrong" opsin in cells without respective membrane enlargements (Kumbalasiri and Provencio 2005). Over time, the cells were provided with intracellular pigment or associated with pigment cells.

The photoreceptive organelles in different flagellate Protista (Gehring 2001, 2004), however, appear to represent one or several alternative structural answers to the selective profit of photosensitivity. The most probable ancestors of Metazoa (based on their primitive monociliar/flagellate collar cells; cf. Rieger 1976) are the Choanoflagellata or Craspedomonadina (Salvini-Plawen 1978). They lack photoreceptive organelles or opsin genes (Plachetzki *et al.* 2007, Suga *et al.* 2008). Therefore, the phylogenetic-structural gap between photoreceptive protists and metazoans additionally points to separate structural formations (morphogeneses) of the photoreceptive equipment. In contrast, the differentiation of photoreception itself, if monophyletic, distinctly points to an earlier process (see above).

POLYPHYLETIC PHOTORECEPTORS IN MOLLUSCA

Our current understanding of the general polyphyletic structural formation and evolution of photoreceptors remains unchanged: the evolutionary pathway of photoreceptors still reflects two different, successive processes. The recognition that the ciliary and rhabdomeric photoreceptive structures are interchangeable morphs, however, alters the premises for detailed homology comparison. Turning to Mollusca, how often within the phylum was such a transcription factor (as a normative induction unit for photoreceptive cell differentiation) convergently co-opted for structural formation (morphogenesis) of photoreceptors?

The wide range of life-styles in molluscs is reflected in a considerable variety of photoreceptive differentiations, from dermal light sensitivity to a rich diversity of ocelli and eyes (Messenger 1991). Larval and/or adult photoreceptors are present in several clades of Testaria (Placophora and Conchifera; cf. Salvini-Plawen 2006). On one hand, there are cerebrally innervated organs, restricted to larvae and organisms with a free head (Gastropoda, Cephalopoda). On the other hand, there are quite a number of "extraocular/ectoptic/extraretinal" organs. In Bivalvia, these are represented by a rich morphological diversity. Following the probable evolutionary history of molluscs (Salvini-Plawen 1990, 2006, Salvini-Plawen and Steiner 1996, Haszprunar 2000), however, they originally lacked any photoreceptors (see Solenogastres, Caudofoveata, Tryblidia, Scaphopoda; cf. Salvini-Plawen 1972, 1980b, 1982, Salvini-Plawen and Mayr 1977). Nonetheless, scaphopods do show dermal sensitivity with shadow responses (see Messenger 1991). Thus, all photoreceptive organs of different lines, including larval ocelli, appear to represent ingroup acquisitions, and no reliable descendance from differentiations in other spiralians can be ascertained.

Larval photoreceptors

Mollusc larvae primarily possess no photoreceptors (Salvini-Plawen 1980b, 1982, in contrast to Bartolomaeus 1992): no ocelli are differentiated in the larvae of Solenogastres, Caudofoveata, Scaphopoda, and protobranch as well as true lamellibranch Bivalvia. The pseudo-trochophoran larvae of Placophora, however, have a pair of ocelli which may persist for some time in the juveniles. Yet, these are post-trochal and laterally innervated (without a homologous equivalent throughout metazoans; Salvini-Plawen 1982, 1988). They have a mixed receptor type of microvilli and of cilia with a somewhat irregular membrane and "a few short microvilli-like projections" (Fischer 1980: 54; see also Rosen *et al.* 1979, Bartolomaeus 1992).

In contrast, the larval ocelli in Bivalvia-Pteriomorpha are cerebrally innervated and may persist in the adults at the first branchial filament. Therefore, a homology had been argued with the cerebral photoreceptors in gastropod veligers (Rosen *et al.* 1978, Bartolomaeus 1992). Four facts contradict this. (1) The most conservative Bivalvia-"Protobranchia" as well as the true lamellibranch bivalves lack ocelli, as mentioned above. (2) Within the Bivalvia (see Fig. 2), larval ocelli are thus restricted to several families of Pteriomorpha (Arcidae to Ostreidae; Pelseneer 1908, Hickman and Gruffydd 1971, Rosen *et al.* 1978, Morton 2001). (3) The larval gastropod photoreceptors are precocious (accelerated) adult organs, not yet differentiated either in the larvae of basal gastropods or in the plesiomorphically common organization of bivalves and gastropods. (4) The Bivalvia, whose pre-pedal body is still covered by the shell, clearly evolved prior to the separation of the free "head" (to which cerebral eyes are correlated). In the latter organizations—the Gastropoda and Cephalopoda—the shell is restricted to the pallio-visceral body. Consequently, a common precursor with "head" and cerebral photoreceptors in Bivalvia (and thereafter accelerated into the larvae) can be excluded (see "Protobranchia"). Accordingly, those larval ocelli have independently evolved only in Pteriomorpha (Pelseneer 1908, Salvini-Plawen 1982). This reflects an apomorphy for this clade (see Fig. 2). The sensory cells of these cup-like ocelli show a microvillous receptor structure with a cilium (Rosen *et al.* 1978), and in *Mytilus edulis* Linnaeus, 1758 larvae they react photo-positively (Bayne 1964).

Among the Gastropoda, the lecithotrophic pseudo-trochophores of the most conservative clade, the stereoglossate Docoglossa (patellogastropods), lack photoreceptors. This is also true for pseudo-trochophores of several Vetigastropoda (*Haliotis*, *Fissurella* Bruguière, 1789) although these later ("pre-veliger"), however, develop photoreceptors, as do most true veligers of advanced gastropods. They represent the precociously advanced (accelerated) organs of the adult organization (Salvini-Plawen 1980b) rather than primary larval organs (Bartolomaeus 1992). The trend to accelerate the organs is obvious in *Haliotis* for example (Crofts 1937, Buckland-Nicks *et al.* 2002), and reflects the prolongation of larval life in the pelagic realm (differentiation of the prototroch to a velum with a second ciliated band, not homologous to a metatroch; Henry *et al.* 2007). In short-term veligers, the photoreceptors are rhabdomeric as in their adults. In long-term (teleplanic) veligers, however, the receptor structure is ciliary and changes during metamorphosis to a rhabdomeric or mixed type (Blumer 1994, 1995, 1996).

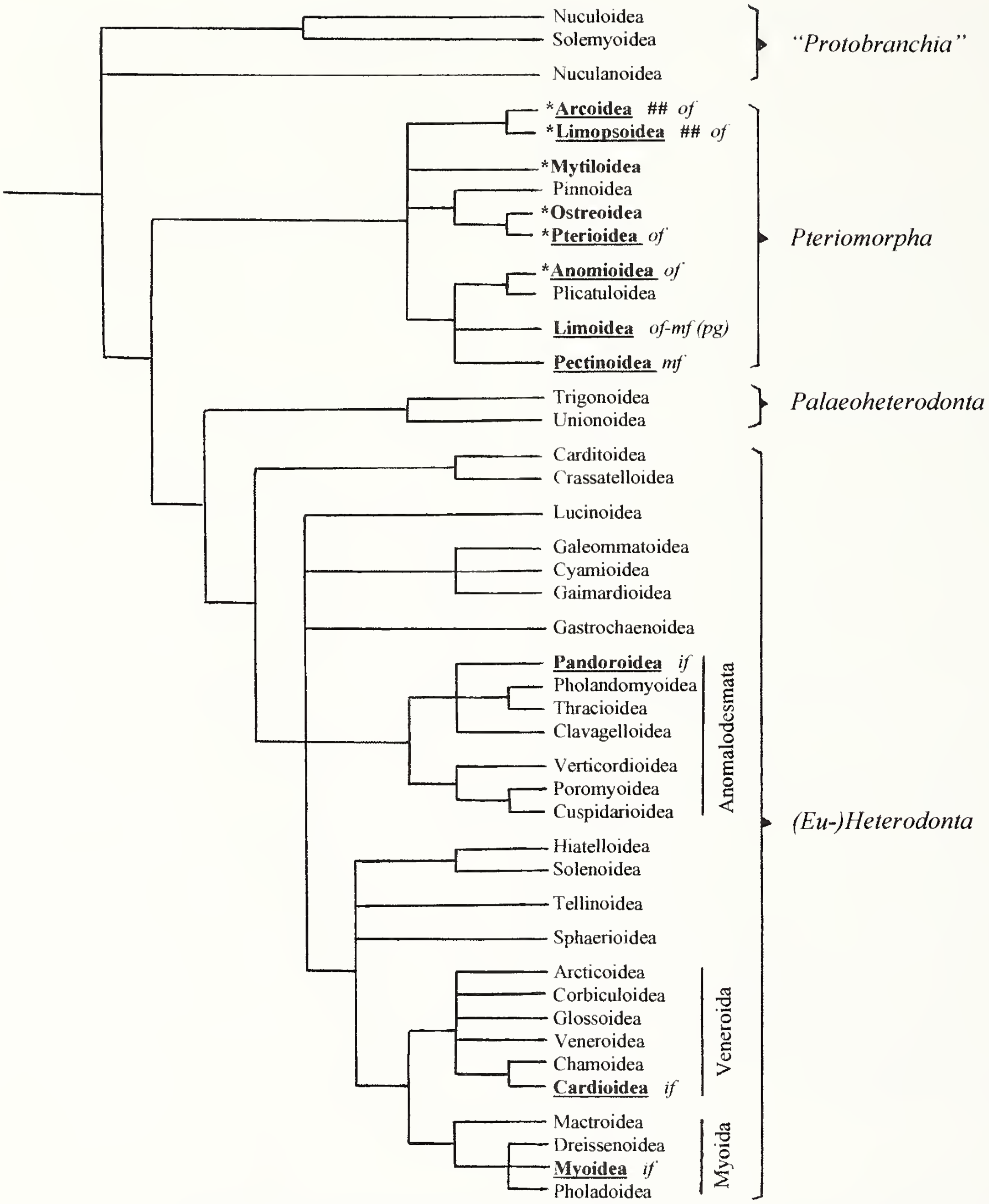
Nuculoidea
Solemyoidea
Nuculanoidea
"Protobranchia"
*Arcoidea ## of
*Limopsoidea ## of
*Mytiloidea
Pinnoidea
*Ostreoidea
*Pterioidea of
*Anomioidea of
Plicatuloidea
Limoidea of-mf (pg)
Pectinoidea mf
Pteriomorpha
Trigonoidea
Unionoidea
Palaeoheterodonta
Carditoidea
Crassatelloidea
Lucinoidea
Galeommatoidea
Cyamioidea
Gaimardioidea
Gastrochaenoidea
Pandoroidea if
Pholandomyoidea
Thracioidea
Clavagelloidea
Verticordioidea
Poromyoidea
Cuspidarioidea
Anomalodesmata
(Eu-)Heterodonta
Hiatelloidea
Solenoidea
Tellinoidea
Sphaerioidea
Arcticoidea
Corbiculoidea
Glossoidea
Veneroidea
Chamoidea
Cardioidea if
Veneroida
Mactroidea
Dreissenoidea
Myoidea if
Pholadoidea
Myoida

Figure 2. Tentative relationships of family groups within Bivalvia (after Steiner and Hammer 2000, Morton 2001, Giribet and Distel 2003), showing differentiation of photoereceptors. **bold**, with photoreceptors; underlined, with pallial photoreceptors; *, with larval/cerebral ocelli; # #, with compound eyes; *if*, at inner mantle fold; *mf*, at middle mantle fold; *of*, at outer mantle fold; *pg*, in periostracum groove (*of-mf*).

Organs in adults

Adult Mollusca differentiate several types of photoreceptive organs. Most exceptional are the aesthetes and shell-eyes in Placophora, but most prominent are the cerebral photoreceptors and eyes in Gastropoda and Cephalopoda. In autobranch Bivalvia (bivalves except for Protobranchia), different types of photoreoreceptors are present at the exposed mantle edge, the latter which serves for general orientation. Such non-cerebral photoreceptive organs are also known in gastropods and cephalopods (see Table 1).

Placophora

Adult Placophora possess a particular type of organ, the aesthete. These are laterally innervated and located dorsally, embedded within the secondarily calcified outer layer (tegmentum) of the eight shell-valves. In addition to other cell types, they include photoreceptors, which in some taxa have also given rise to advanced shell-eyes. The photoreceptive cells of the aesthetes are generally microvillous (rhabdomeric; Fischer 1988). In *Acanthochiton fascicularis* (Linnaeus, 1767), however, the aesthetes of the lateral fields of the valves differ by including sense cells with ciliary lamellate receptors (Fischer 1979). The shell-eyes (with lenses) also possess rhabdomeric receptor structures, whereas their marginal cells have ciliary receptors (Boyle 1969). In both these cases, the different receptors probably perform dissimilar functions (*e.g.*, for polarized light?). The extraordinary sensory organs within the valves of *Cryptoplax mystica* Iredale and Hull, 1905 (see Currie 1992) should also be mentioned although their function is still uncertain. They are surrounded by pigmented tissue and demonstrate a highly aberrant structure consistent with a photoreceptive and/or a balance organ.

Gastropoda

Cerebral photoreceptors in Gastropoda are everse but differentiated in various degrees. The structures range from open eye-pits such as in Patellidae (Docoglossa) or in Neritilidae (Neritopsina; Kano and Kase 2002) to the well-equipped lens-eyes in higher Caenogastropoda and Euthyneura (Salvini-Plawen and Mayr 1977). The receptor structure is generally rhabdomeric. Some species, however, show a mixed rhabdomeric and ciliary type: *Haliotis (Nordotis) discus* Reeve, 1846, *Viviparus viviparus* (Linnaeus, 1758), *Lacuna vincta* (Montagu, 1803) [=*Lacuna divaricata* (Fabricius, 1780)], possibly *Fartulum orcutti* (Dall, 1885), *Aporrhais pespelicani* (Linnaeus, 1758), *Aplysia punctata* (Cuvier, 1803) (see Howard and Martin 1984, Bartolomaeus 1992, Blumer 1995, 1996, Zhukov *et al.* 2006). In the ho-

Table 1. Polyphyletic evolution of photoreceptors, ocelli, and eyes in Mollusca (13 lines).

Taxon	Photoreceptors	Structure	Reference (see also text)
Solenogastres	—		Salvini-Plawen 1982
Caudofoveata	—		Salvini-Plawen 1982
(1) Placophora (larvae)	laterally innervated larval ocelli	mixed	Fischer 1980, Bartolomaeus 1992
(2) Placophora (adult)	aesthetes in general	rhabdomeric	Fischer 1988
Placophora (adult)	*Acanthochiton* lateral aesthetes	ciliary	Fischer 1979
Placophora (adult)	extrapigmental shell-eyes	rhabomeric	Boyle 1969
Placophora (adult)	shell-eye marginal cells	ciliary	Boyle 1969
Tryblidia	—		Salvini-Plawen 1982
(3) Gastropoda in general, adult	cerebral photoreceptors	rhabdomeric or mixed	Blumer 1996
Gastropoda-Heteropoda,	adult cerebral eyes	ciliary or mixed	Blumer 1999
Gastropoda, telephanic veligers	cerebral photoreceptors	ciliary	Blumer 1996
(4) Caenogastropoda: *Cerithidea*	pallial eye	ciliary	Rogge 1987
(5) Gastropoda-Anaspidea: *Aplysia*	abdominal neurons	diverticular	Messenger 1991
(6) Gastropoda-Onchidiidae	dorsal eyes	ciliary	Katagiri *et al.* 1985
Gastropoda-Onchidiidae	lens cells of dorsal eyes	rhabdomeric	Katagiri *et al.* 1985
(3?) Siphonopoda (cephalopods)	cerebral photoreceptors	rhabdomeric	Messenger 1991
(7) Siphonopoda	photosensitive vesicles	rhabdomeric	Messenger 1991
Scaphopoda	—		Messenger 1991
(8) Bivalvia-Pteriomorpha	cerebral/larval ocelli	rhabdomeric	Rosen et al. 1978
(9) Bivalvia-Arcoida	compound eyes at mantle edge	ciliary	Nilsson 1994
(10) Bivalvia-Arcoida	pigment-cup ocelli at mantle edge	rhabdomeric	Nilsson 1994
Bivalvia-Limoidea	eyes at mantle edge	mixed	Steiner 2001
Bivalvia-Pectinoidea	eyes with two retinae at mantle edge	rhabdomeric (proximal) ciliary (distal)	Steiner 2001
(11) Bivalvia-Pandoroidea	eyes with one or two retinae at siphons	both retinae ciliary	Adal & Morton 1973, Prezant 1984
(12) Bivalvia-Cardioidea	eyes at siphons	ciliary or mixed	Salvini-Plawen 1982
(13) Bivalvia-Myidae	phaosomes embedded in siphons	?rhabdomeric?	Light 1930

lopelagic Heteropoda, the retina of some species is subdivided in adults into an anterior segment with rhabdomeric receptor cells and a posterior segment with ciliary structure. Other heteropod species exclusively exhibit a ciliary type of cerebral photoreceptor, as do all heteropod veligers (Blumer 1998, 1999).

Apart from these cerebral organs, there is a clearly non-homologous pallial eye in the caenogastropod *Cerithidea scalariformis* Say, 1825. This has ciliary retinal cells that allow light/shadow reactions (Rogge 1987).

The photosensitive neurons in the abdominal ganglion in *Aplysia californica* J. G. Cooper, 1863 also deserve mention (see Messenger 1991). They consist of two receptor cells of the diverticular type (see above) which respond to light by hyperpolarization; each possesses intracellular organelles (lipochondria) with two orange-red photopigments (Andresen and Brown 1982).

The Onchidiidae—a family of the Gymnomorpha (within Aeropneusta the sister-group to Eupulmonata)—are also noteworthy. Several members possess, apart from cerebral eyes (stalked, rhabdomeric), dorsal papillae with up to seven eyes. These dorsal mantle eyes show an inverse retina as well as two lenses above each other (Stantschinsky 1908) and are pleurally innervated via pallial nerves. The fine structure reveals particular features: in *Onchidium verruculatum* Cuvier, 1830, the retina consists of cells with ciliary receptors which hyperpolarize with an "off-response" (Katagiri *et al.* 1985). The upper, unicellular lens is also photoreceptive but with a dense brush of microvilli (rhabdomeric type) and depolarizes with "on-response". In addition, *Onchidium* possesses single dermal photoreceptive cells, isolated or in small clusters, devoid of associated pigment; they have dense microvilli (rhabdomeric type) and depolarize with an "on-response".

Cephalopoda

The typically everse cerebral eyes of Cephalopoda initially differentiate a rudiment of the retina whose cells have a root-less cilium and microvilli. This subsequently develops into a rhabdomeric receptor structure like most Gastropoda (Yamamoto 1985, Muntz and Wentworth 1987, Messenger 1991). The free head and other synapomorphic characters in cephalopods and gastropods (Salvini-Plawen 1990, Salvini-Plawen and Steiner 1996, Haszprunar 2000) suggest the cerebral eyes of both groups (= Visceroconcha) as being homologous although evolved to different complexity. *Nautilus* Linnaeus, 1758, exhibits simple pin-hole organs, whereas the Coleoida possess highly differentiated lens-eyes. Additional photoreceptors are the so-called photosensitive vesicles, viz. the epistellar bodies in Octobrachia and the parolfactory vesicles in Decabrachia (Salvini-Plawen and Mayr 1977, Messenger 1991, Cobb and Williamson 1998, Parry 2000). These "extraocular/ectoptic/extraretinal" organs are provided with microvillous receptor cells, contain the visual pigment rhodopsin, and show a depolarizing reaction to light.

Bivalvia

In Bivalvia, six different lines of photoreceptive organs may be recognized (Salvini-Plawen and Mayr 1977, Morton 2001; Fig. 2). One of these (1) includes cerebral ocelli, whereas five lines (2)-(6) are "extraocular/ectoptic/extraretinal" photoreceptors that originate at the mantle rim(s). The border of the mantle, including the siphons, appears to have acquired a general light sensitivity (see "dermal" light sense, above; Kennedy 1960, Mpitsos 1973, Morton 2001). This gave rise, at different mantle folds and in various groups (Morton 2001; see Fig. 2), to photoreceptive organs, all innervated by pallial nerves from the visceral ganglia.

(1) The cerebrally innervated, cup-like ocelli at the first branchial filament represent the larval ocelli (see above). In *Striarca lactea* (Linnaeus, 1758) they are located at the posterior labial palp (Thiele 1902: 380 and figs. 145-146). These organs persist for various times also in the adult animals. They occur in representatives of several pteriomorph families (Pelseneer 1908, Rosen *et al.* 1978, Morton 2001) and some may be provided with a simple lens (Morton 1978).

(2) A type of compound eye originates at the mantle edge of arcoidan representatives (cf. Morton 2001). This includes Arcidae, Glycimeridae (*Pectunculus* in Land 1984 = *Glycimeris* Da Costa, 1778), Cucullaeidae (*Cucullaea* Lamarck, 1801), and Limopsidae (*Philobrya* Carpenter, 1872). Such eyes parallel the compound ocelli of sabellid polychaetes as well as the complex eyes of arthropods (cf. Nilsson 1994, Land and Nilsson 2006). Each "ommatidium" lacks a real lens and represents an unusually modified ciliary photoreceptive cell accompanied by a supporting pigment cell (Levi and Levi 1971, Nilsson 1994). Each sensory cell is covered by a lateral expansion with microvillosities of the supporting cell and shows an inversed polarity: the nucleus is positioned distally and the median membrane enlargements of the laterally located cilia are arranged above each other in the basal third of the cell. They react to a decrease in light intensity.

(3) In Pteriomorpha, there are additional photoreceptive organs at the mantle edge (see Fig. 2) which may co-exist with compound eyes or even cerebral ocelli; this underlines that this clade as adapted to the eulittoral zone. They show a wide morphological range in configuration and structure. Examples include the simple cap-shaped eyespots in Pteri-

oidea (*e.g., Isognomon* Lightfoot, 1786; Morton 2001), simple everse pigment-cup pits in Arcoidea (*e.g., Arca* Linnaeus, 1758; Nilsson 1994), pinhole-like organs in Limoidea (*e.g., Lima* Bruguière, 1797; Hesse 1900 and below), everse lens eyes in Limoidea (*e.g., Ctenoides* Mörch, 1853; Morton 2001) as well as on the outer surface of the mantle in Anomioidea (*e.g., Enigmonia* Iredale, 1918; Morton 2001), and the unusual organs with lens and two retinae in Pectinoidea (Pectinidae, Spondylidae).

With respect to the receptor structure among Pteriomorpha, except for Pectinoidea, only the pigment-cup photoreceptive organs in Arcidae have been investigated (*Arca, Barbatia* Gray, 1842, *Anadara* Gray, 1847; Nilsson 1994). More ultrastructural studies in other Pteriomorpha are needed. The organs of Arcidae are rhabdomeric. In the study by Steiner (2001), three additional species were ultrastructurally investigated: the two pectinids *Chlamys varia* (Linnaeus, 1758) and *Pseudamussium peslutre* (Linnaeus, 1771) as well as the limoidean *Lima lima* (Linnaeus, 1758) [= *Lima squamosa* Lamarck, 1891]. The eye structure of the two pectinids (Fig. 3) is similar to that of *Pecten maximus* (Linnaeus, 1758) (see Barber *et al.* 1967), *i.e.*, an inverted retina of rhabdomeric receptors as well as a distal retina of everse ciliary receptor cells. The organs possibly function as concave mirror eyes (Land 2000). In both species, the upper, everted retina shows cells with distally flattened cilia arranged in whorls (Fig. 4A). Central microtubules and rootlets are lacking. The two species differ from *Pecten* in that the proximal rhabdomeric receptor cells have numerous long ciliary rootlets (Figs. 4B, 5). The cornea cells are also considerably taller at the center than peripherally (see also Morton 2001 for *Patinopecten yessoensis* (Jay, 1857) and *Chlamys pusio* (Linnaeus, 1758)). Another major difference is in the arrangement of the proximal retina. Whereas in *Chlamys varia* the rhabdomeric receptive regions of the long sensory cells form a closed layer below the distal retina, their nucleus-containing regions are fairly peripherally arranged. In contrast, the proximal retina of *Pseudamussium peslutre* does not form a continuous layer below the distal retina; rather, the ciliary retina, which is smaller in diameter, is only peripherally underlain by the rhabdomeric receptive regions (Fig. 3). Thus, the proximal retina in *Pseudamussium* merely forms an incomplete, ring-like layer centrally without receptors. In the eye axis, only the distal, ciliary retina probably functions for photoreception.

The distally open photoreceptive organs of *Lima lima*, without cornea or lens, are formations of the periostracal groove between outer and middle mantle folds (Fig. 6). As already roughly described by Mpitsos (1973) for *Lima scabra* (Born, 1778), rhabdomeric and ciliary sections are separable in the retina; Nasi (1991) figured isolated rhabdomeric sensory cells with a distal microvillous lobe. Similarly, the retina of *L. lima* is divided into two regions, the proximal rhabdomeric and pigmented cells on the one hand, and distal ciliary receptor cells on the other hand. The latter also show some microvilli. In *L. lima*, however, the transition from rhabdomeric to ciliary receptors is gradual. The rhabdomeric cells show several cilia at the "neck" leading to the bulbous receptive portion (Fig. 7), thus perhaps representing a mixed type. The ciliary receptors lack rootlets as well as central microtubules, and the vast enlargements of their ciliary membrane surfaces fill the eye cavity ("vitreous mass", Fig. 8; compare *Tridacna* Bruguière, 1797, below). Part of the middle mantle fold, above the periostracum-secreting cells, shows cells with large void spaces and few organelles, thus representing a "window" for the light (Fig. 6).

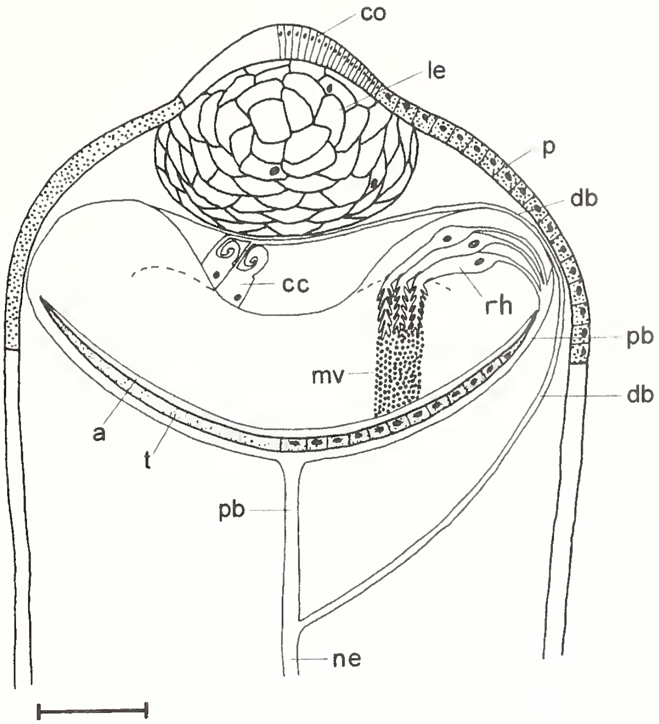

Figure 3. *Pseudamussium peslutre* (Bivalvia). Diagram of a longitudinal section of the eye. a, argentea; cc, ciliary retina cell; co, cornea; db, distal branch of optic nerve; le, lens; ne, optic nerve; p, epidermal pigment ring; pb, proximal branch of optic nerve; rh, rhabdomeric sensory cell; t, pigmented tapetum cells. The transition from the cell bodies (proximal retina) to the rhabdomeric rod is marked by desmosomes (dotted line). Scale bar = 100 μm. (From Steiner 2001).

No decision can be made as to a homology versus a homoiology of all these photoreceptive organs at different mantle folds between the pteriomorph family groups (see also Morton 2001, 2008). Particularly, no known configuration of photoreceptive organ forms a transition to the ex-

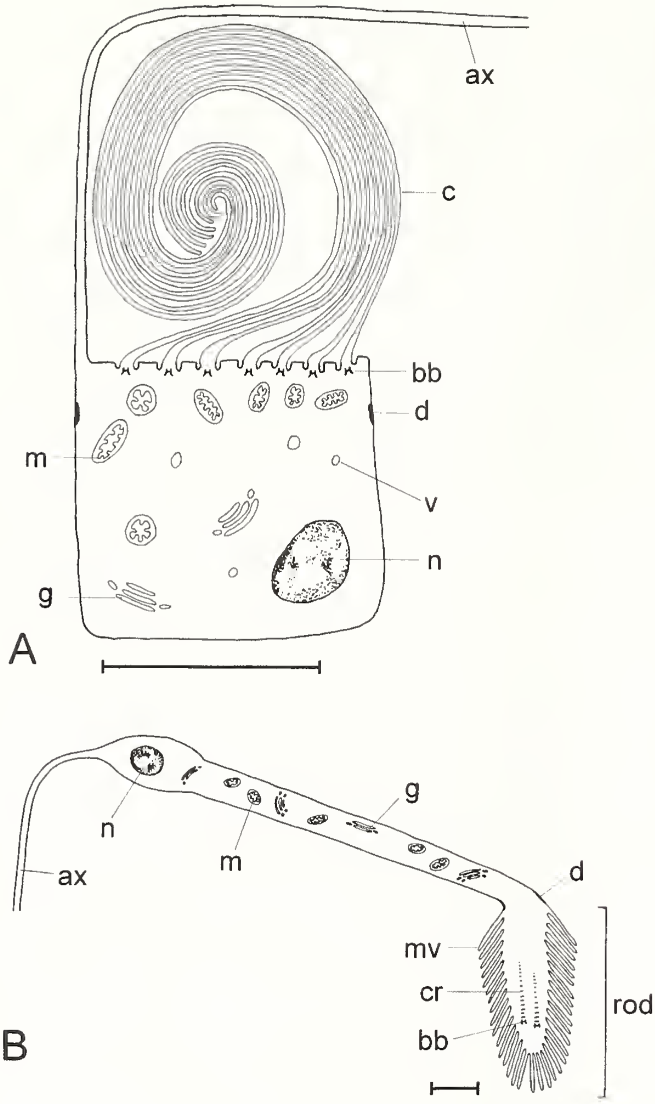

Figure 4. *Chlamys varia* (Bivalvia). A, Ciliary sensory cell of the distal retina. Scale bar = 5 μm. B, Rhabdomeric sensory cell of the proximal retina. Scale bar = 10 μm. a, axon; bb, basal body; c, cilia; cr, ciliary rootlet; d, desmosomes; g, Golgi stack; m, mitochondrion; mv, microvilli; n, nucleus; v, vesicle. (From Steiner 2001).

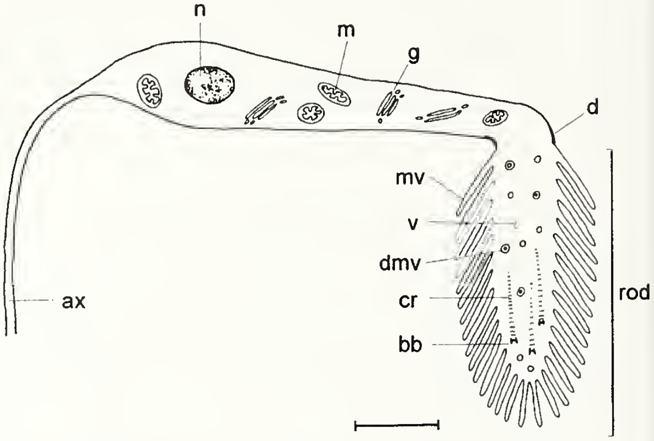

Figure 5. *Pseudamussium peslutre* (Bivalvia). Rhabdomeric sensory cell of the proximal retina. ax, axon; bb, basal body; cr, ciliary rootlet; d, desmosome; dmv, double-membraned vesicle; g, Golgi stack; m, mitochondrion; mv, microvilli; n, nucleus; v, vesicle. Scale bar = 10 μm. (From Steiner 2001).

traordinary eyes of Pectinoidea. The earlier reference (Salvini-Plawen and Mayr 1977, Salvini-Plawen 1982) to a morphological sequence has been questioned (Morton 2001), *i.e.*, that an "accessory receptor" of inverse eyes (such as in Cardiidae, below) could have been modified to the distal retina in Pectinoidea. The very distant relationship of the representatives (Veneroidea versus Pteriomorpha; see Fig. 2) and the independent formation of two retinae in *Laternula* Roeding, 1798 (Anomalodesmata, below) renders the hypothesis speculative. The existence of an "accessory organ" associated with the photoreceptors also in some Pteriomorpha (*e.g.*, *Philobrya* Carpenter, 1872, *Enigmonia*; see Morton 2001), however, does not exclude such a structural sequence as a differentiation model for the eyes of Pectinoidea. An alternative view (see also Steiner 2001) refers to the more closely related Limoidea (see Fig. 2), whose retina shows two sections. A major shift and concentration of their ciliary cells (distal section) towards the light-exposed side, followed by their separation and medial inversion, would form a primordial distal retina. This would be followed by later reversion to everse cells (compare organogenesis in pectinids; Hesse 1908, Küpfer 1916). In the proximal section, a concentration of the pigment cells mid-ventrally (eventually becoming a tapetum) and separation from the lateral (rhabdomeric) receptive cells would favor a medially directed inversion of the latter. This would then represent a peripheral proximal retina such as in *Pseudamussium peslutre*.

(4) Within Anomalodesmata-Pandoroidea-Pandoridae, the unusual pallial photoreceptive organs in *Laternula* species [= *Anatina* in Pelseneer 1908, footnote] are situated on tentacles around the siphons. The eyes with lens have two retinae above each other, both built up (in contrast to Pectinoidea) of everse cells with ciliary receptor structure. They are surrounded by a cup of pigment cells delimited by a sclerotic coat (Adal and Morton 1973). Similar to the accessory receptors in Cardiinae and Tridacninae, an eye appendage associated with the optic nerve is present; this includes a vertical channel with a bundle of long cilia at its base and apparently represents a mechanoreceptor (Morton 2001).

Other photoreceptive organs are reported in Pandoroi-

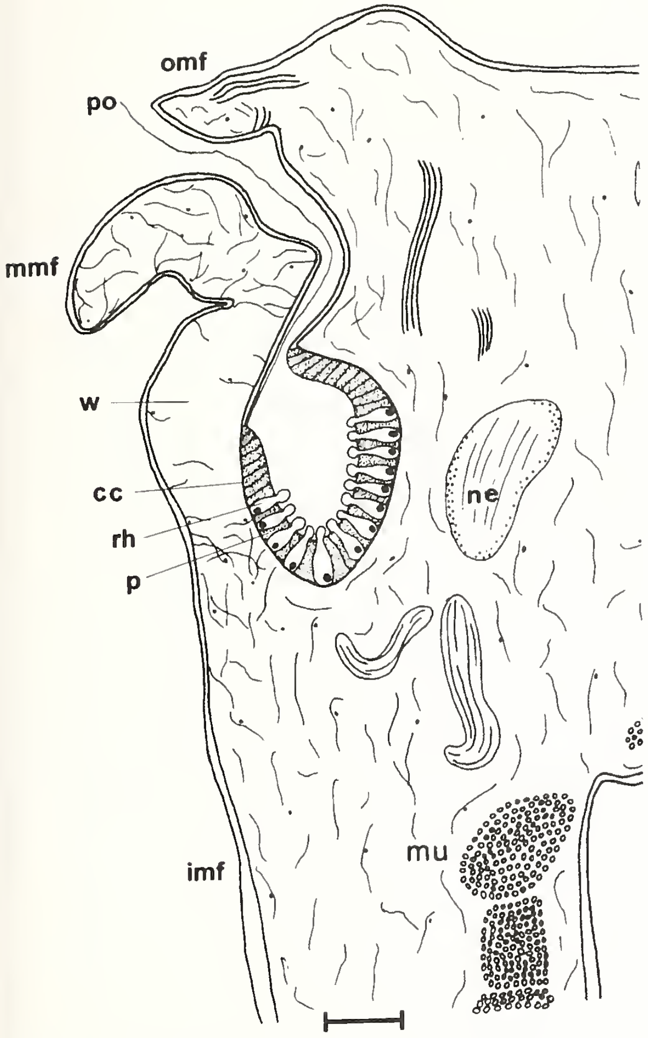

Figure 6. *Lima lima* (Bivalvia). Diagram of a longitudinal section of the mantle folds with the eye cup. cc, ciliary sensory cells; imf, inner mantle fold; mmf, middle mantle fold; mu, muscle; ne, nerve; omf, outer mantle fold; p, pigment cell; po, periostracum; rh, rhabdomeric sensory cell; w, "window". Scale bar = 100 μm. (From Steiner 2001).

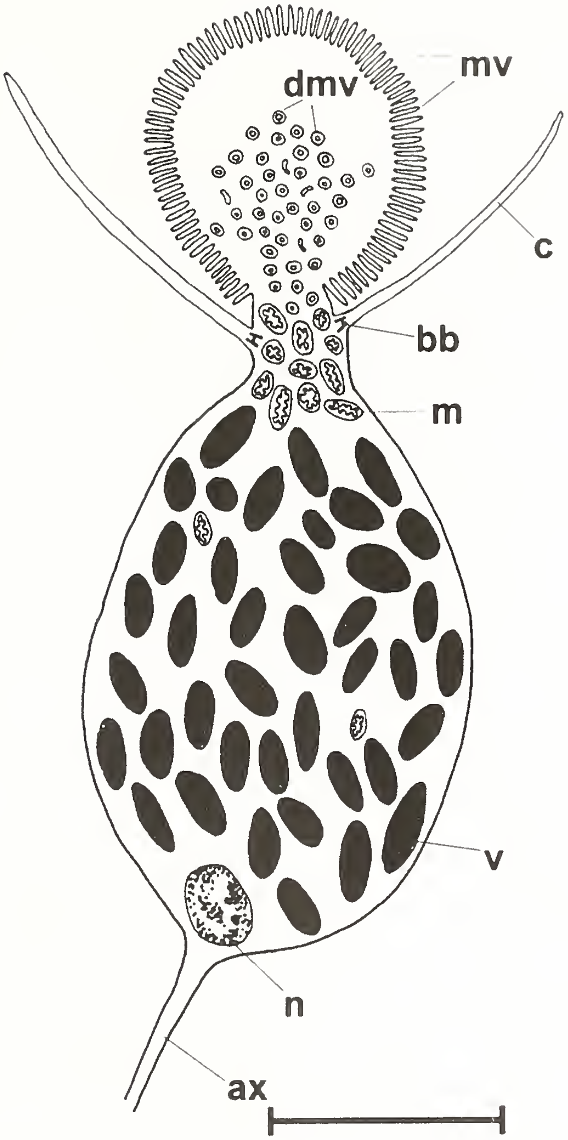

Figure 7. *Lima lima* (Bivalvia). Diagram of a rhabdomeric sensory cell of the proximal eye cup. bb, basal body of cilium, c, cilium; dmv, double-membraned vesicle; m, mitochondrion; mv, microvilli; n, nucleus; v, vesicles. Scale bar = 20 μm (from Steiner 2001).

dea-Lyonsiidae by Prezant (1984) for *Lyonsia hyalina* (Conrad, 1831) along the exhalant siphon. These lens-eyes have pigment cells and they have ciliary sensory cells with the membrane-enlargements in whorls. Proximally, these whorls form concentric rings similar to those in *Laternula* (above).

(5) Among Veneroida, members of the more closely related Cardiinae and Tridacninae (Cardioidea) show photoreceptive organs at the siphonal tissues; in Cardiinae a large number is present at the tips of tentacles. In *Cerastoderma edule* (Linnaeus, 1758) the eyes are more or less inverted without a lens and the receptor cells (ciliary type) are enclosed in a cup of reflecting cells (Barber and Wright 1969). The inner epithelium of the tentacle, basal to the eye cup, contains pigment granules that presumably also serve as a reflecting surface (Morton 2001). In addition, an "accessory receptor organ" at the tip of the tentacle is associated with the optic nerve. Its receptor cells bear numerous unmodified cilia and microvilli.

In *Tridacna*, the abundant eyes are everse and the receptor cells are of the ciliary type. The mass of the microvillous membrane-enlargements of the cilia may also function as a lens ("vitreous mass"; see *Lima*, above). No cup of

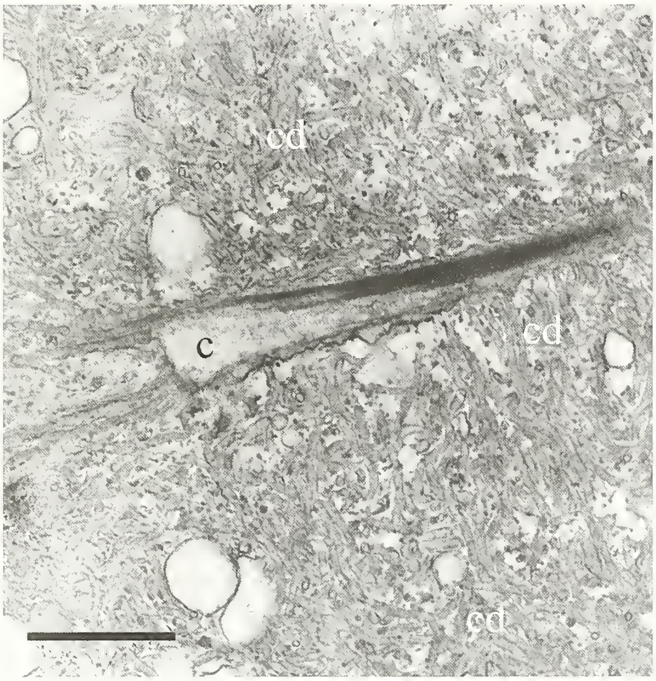

Figure 8. *Lima lima* (Bivalvia). Ultrastructural section of a ciliary sensory cell of the distal area of the retina with tube-like diverticles of the ciliary membrane filling the eye cup ("vitreous mass"). c, cilium; cd, tube-like diverticles. Scale bar = 1 µm. (From Steiner 2001).

pigment cells is present, but surrounding layers of olive-green zooxanthellae may have a reflective function (Frankboner 1981). A ciliated "accessory receptor" is associated with the optic nerve.

(6) Within Myoida, thousands of pear-shaped, pigment-less cells have been reported and described in the siphons of *Mya arenaria* Linnaeus, 1758 (Light 1930). They are differentiated just beneath the inner epithelium of both (incurrent and excurrent) siphons. No fine structural investigation is available, but these cells, which have axons to the siphonal nerves and an enclosed optic organelle, are devoid of associated shading pigment. They nonetheless react to an increase in light intensity with contraction of the joined siphons. These pear-shaped cells thus parallel the phaosome cells in Clitellata in both structure and function (see Purschke 2003), the latter representing unicellular photoreceptors.

FINAL CONCLUSION

The proposed evolution of photoreceptors according to two different, successive processes is compatible with the general expression of transcription factor genes as well as with the rich morphological diversity of the photoreceptive organs throughout the metazoans. Earlier representations with respect to this polyphyly are now modified: the structural types of the epidermal photosensitive receptors (ciliary, rhabdomeric) are now considered to be mere morphs, obviating the need for arguments on homology or non-homology. The homology identification of photoreceptors must, therefore, follow other precise criteria (relationship, continuity, structural and/or developmental intermediates, identical gene regulatory network). According to such criteria, evolutionarily independent structural differentiation (morphogenesis) of photoreceptors in Mollusca can be proposed for at least thirteen lines (Table 1), irrespective of potential multiple homoiology of pallial organs within pteriomorph Bivalvia (Fig. 2).

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Primary inhibition by light: A unique property of bivalve photoreceptors*

Lon A. Wilkens

Center for Neurodynamics and Department of Biology, University of Missouri-St. Louis, One University Boulevard,
St. Louis, Missouri 63121, U.S.A., wilkensl@umsl.edu

Abstract: Bivalve molluscs are known for shadow responses involving closure or retraction of the siphon and valve adduction. In representative genera [*Spisula solidissima* (Dillwyn, 1918), *Mercenaria mercenaria* (Linnaeus, 1758), *Lima scabra* (Born, 1778)], the pallial nerves contain photosensitive fibers that exhibit physiological shadow responses. These photoreceptors are inhibited by light but trigger an excitatory burst of spikes to a shadow, the off-response. Equivalent responses are seen in bivalve eyes, *e.g.*, in optic fibers from the siphon tentacle eyes of *Cardium edule* (Linnaeus, 1758) and the mantle eyes of scallops (Pectinidae) and file clams (Limidae). In scallops, they form a distal retinal layer of ciliary receptors, distinct from a proximal microvillar layer that is excited by light. In off-receptors (ciliary), light inhibition is the result of a hyperpolarizing receptor potential with spikes generated on the rebound depolarization at dimming. In contrast, the proximal on-receptors are excited by light with spikes generated by depolarizing receptor potentials. The inhibitory effect is first-order, *i.e.*, a direct response to light, as is excitation for the proximal receptors. With separate retinas and the absence of synaptic contact, these are the primary receptors. Aside from *Pecten* Müller, 1776 and *Lima* Bruguiére, 1797, the only other bivalve eyes in which receptor potentials have been investigated are those of the giant clam *Tridacna maxima* (Röding, 1798). Here there are two types of hyperpolarizing, light-inhibited primary receptors, one of which generates spikes at light offset, the other non-spiking. The inhibitory response is universal in bivalve photoreception, unique among the eyes of invertebrates, but similar in polarity to chordate photoreceptors although the ionic mechanisms are different. Receptor physiology is discussed relative to image formation in bivalve eyes.

Key words: shadow response, mantle, retina, *Pecten*, *Tridacna*

The cast of a shadow onto benthic and/or sessile organisms commonly triggers some form of withdrawal into a shell or burrow. Such is the case for most shallow, subtidal bivalve molluscs. In bivalves, this 'shadow response' involves a variety of movements including siphon closure, retraction of the siphon or mantle, and shell adduction (Wenrich 1916). For most bivalves this response does not require eyes. Rather, the mantle tissues themselves are sensitive to light as confirmed in pallial nerve recordings from a number of species (Kennedy 1960, Mpitsos 1973, Wiederhold *et al.* 1973). However, a few bivalve families have added functional eyes ectopically around the shell-mantle edge (Morton 2001). These constitute a well-developed visual system for motion sensitivity that triggers sight reactions independent of direct shadowing of the animal (Nilsson 1994). Most prominent are the distinctive blue eyes of the scallop (Fig. 1), visual organs that have attracted the attention of classical morphologists (Dakin 1910, 1928) to present-day neuroscientists. Morphology and optics have been examined in detail in the eyes of *Pecten maximus* (Linnaeus, 1758) (Dakin 1910, 1928, Land 1965, Barber *et al.* 1967), recently in *Amusium balloti* (Bernardi, 1861), *Argopecten irradians irradians* (Lamarck, 1819), *Chlamys hastate* (Sowerby, 1842), *Chlamys rubida* (Hinds, 1845), *Spondylus americanus* (Herman, 1781), and *Crassodoma gigantea* (Gray, 1825) (Speiser and Johnsen 2008), *Cardium edule* (Linnaeus, 1758), and *Tridacna maxima* (Röding, 1798) (Barber and Land 1967, Kawaguti and Mabuchi 1969, Land 2003, Stasek 1966) to evaluate bivalve vision. Here, physiological properties are examined with emphasis on the unique inhibitory response to light in bivalve photoreception and its role in behavior and visual function.

Light inhibits sensory neurons in the mantle and eyes

Bivalves, alone among the non-chordate invertebrates, have adopted a near-universal inhibitory response to light, a physiological mechanism suitably adapted for the shadow response. Termed 'primary inhibition', activity in photosensory neurons is suppressed by the absorption of light independent of synaptic interactions at the receptor level and therefore a first-order response. Photoreception in chordates is also inhibitory, hyperpolarizing by convention, but with contrasting mechanisms to be considered later. Light inhibition was first observed in optic nerve recordings from scallop eyes (Hartline 1938). Upon dimming these same fibers produce vigorous bursts of action potentials or spikes, the 'off-response'. However, inhibition is also characteristic of the dermal light response in the bivalve mantle. For ex-

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Figure 1. *Argopecten irradians taylorae* (Petuch, 1987) in its shallow-water, turtle grass habitat, St. Joseph Bay, Florida.

ample, the siphonal nerves of the lamellibranchs *Mya arenaria* (Linnaeus, 1758), *Mercenaria (=Venus) mercenaria* (Linnaeus, 1758), and *Spisula solidissima* (Dillwyn, 1817) each contain neurons whose spontaneous activity is inhibited by light. In *Spisula* this "simple" photoreceptor system (Kennedy 1960) consists of a single pair of siphonal neurons leading to the visceral ganglion where the motor response for siphon retraction is generated. In these neurons, the light response has been characterized as a dual inhibitory-excitatory process involving different photopigments. The response is modeled as the sum of a low-threshold, short-wavelength inhibition and a long-wavelength excitation responsible for the off-response burst of impulses (Fig. 2). The photopigments, as yet unidentified, are assumed to coexist within the neuron since the light response does not originate from presynaptic receptors. Likewise, photosensory neurons in the siphon nerves of the hard shell clam *M. mercenaria* are inhibited by light, with spikes elicited only in response to dimming (Wiederhold *et al.* 1973). Membranous whorls in the distal portions of these neurons indicate, again, that these neurons contain photopigments and respond directly to light. Mantle neurons in the file clam *Lima scabra* (Born, 1778) also generate off-responses independent of light sensitivity in the eyes (Mpitsos 1973).

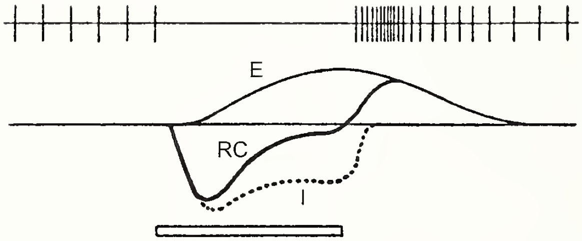

Figure 2. Model of primary inhibition in *Spisula* siphonal neuron. Light (bar) evokes a low-threshold light intensity inhibition (I) and delayed, long-lasting excitation (E) whose sum is the receptor potential (RC) that accounts for the inhibition of spiking and the burst of action potentials at light offset. The inhibitory effect is hyperpolarizing and the excitatory effect depolarizing. After Kennedy (1960).

Light inhibition in the scallop eye was initially attributed to synaptic interactions in the retina (Hartline 1938), reminiscent of the lateral inhibitory synapses among *Limulus polyphemus* ommatidia well known as the basis for contrast enhancement (Hartline and Ratlliff 1958). This interpretation grew out of the fact that the scallop retina contains a dual layer of photoreceptor cells (Fig. 3). Inhibition and the corresponding excitatory off-response are properties of the distal layer, whose ciliary photoreceptors and axons form the distal ramus of the optic nerve. Optic fibers from the proximal, rhabdomeric layer form the proximal ramus and respond to light with an excitatory 'on-response'. Although Hartline favored the interpretation that the proximal receptors were the source of excitation for the off-response in the

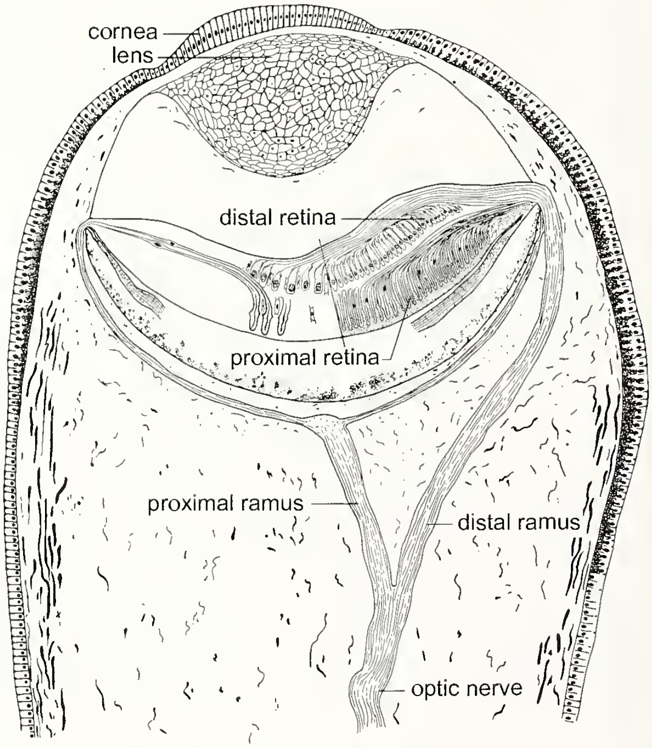

Figure 3. Diagrammatic representation of *Pecten* eye. Modified from Dakin (1928).

distal receptors, the latter equivalent to vertebrate retinal ganglion cells, he also considered the possibility that distal receptors represented a new type of cell, "excited only by the removal of a stimulating agent" (p. 477). The latter interpretation, that the distal receptors are a new type of cell, is now accepted, both for distal receptors in the eye and for photosensory fibers in the mantle. In the eye, ultrastructural examination of the retina has since revealed no evidence for synaptic connections either between proximal and distal layers or among neighboring photoreceptors (Barber *et al.* 1967), thereby ruling out synaptic interactions as the basis for inhibition by light. Unequivocal segregation of proximal and distal responses has also been confirmed by cutting the distal ramus, thereby eliminating off-response activity in the optic nerve (Hartline 1938, Land 1966). With minor differences in spontaneous activity the visual responses are equivalent for all species of scallop. As in scallops, the eyes of *Lima scabra* have a dual retina and on- and off-responses in the respective optic fibers of proximal and distal origin (Mpitsos 1973). Also, ciliary receptors that resemble those in the scallop distal retina are present in the tentacle eyes of *Cardium edule* and spiking in their optic fibers occurs only in response to a decrease in light intensity (Barber and Land 1967).

The inhibitory effect of light in the distal (ciliary) photoreceptors of scallops, along with bivalve siphonal nerves, is unusual considering that a light stimulus to the eyes of most animals is excitatory and triggers bursts of impulses in optic nerve fibers, as in the excitatory on-response in the scallop proximal (rhabdomeric) photoreceptors. An excitatory response, to light or any other sensory stimulus is generated by a depolarizing receptor potential, a reduction in membrane potential toward spike threshold. Impulses arise in response to these depolarizing currents, either at the spike initiation zone of photosensory neurons or in presynaptic non-spiking retinal cells. Hyperpolarization, in contrast, increases the membrane potential away from threshold levels exerting a stabilizing, spike-inhibiting influence. Excitatory, depolarizing receptor potentials in response to light are characteristic of all non-chordate invertebrate eyes examined so far, with bivalve eyes the only exception. Representative examples, based on intracellularly recorded receptor potentials, include annelids (Walther 1965, Fioravanti and Fuortes 1972), insects (Naka 1961), gastropod (Dennis 1967) and cephalopod molluscs (Tomita 1968), and arthropods, as first demonstrated in the horseshoe crab *Limulus polyphemus* (Hartline *et al.* 1952, Fuortes 1959). Each of these 'excitatory' examples represents a phylum or taxon (annelid, gastropod, cephalopod, arthropod) with rhabdomeric photoreceptors. Photosensitive neurons in the arthropod central nervous system are also excitatory, *e.g.*, the crayfish caudal photoreceptor, a photosensory interneuron in the 6th abdominal ganglion discovered by Prosser (1934) and later shown to be depolarized by light (Wilkens and Larimer 1972). However, exceptions to the bivalve-only inhibitory effect exist if one considers extraocular photosensitivity, *e.g.*, gastropod CNS neurons are inhibited and/or hyperpolarized by light (*Aplysia californica* (Cooper, 1863), Brown and Brown 1973; *Onchidium verruculatum* (Cuvier, 1830), Goto and Nishi 2002; see review of extraocular photosensitivity by Yoshida 1979). In most instances these neurons are neither ciliary nor rhabdomeric.

In bivalves, receptor potentials obtained from intracellular recordings were first observed in photoreceptors from scallop eyes (Toyoda and Shapely 1967, Gorman and McReynolds 1969). Receptor potentials in the distal layer are hyperpolarizing in response to light (Fig. 4B), consistent with primary inhibition and spike suppression previously established for these receptors. At light offset the membrane potential rapidly depolarizes generating the off-response burst of spikes. In contrast, the proximal photoreceptors are depolarizing (Fig. 4A), generating spikes at light onset. Similar hyperpolarizing (inhibitory) and depolarizing (excitatory) responses have been reported for the corresponding retinal receptors in *Lima scabra* (Mpitsos 1973).

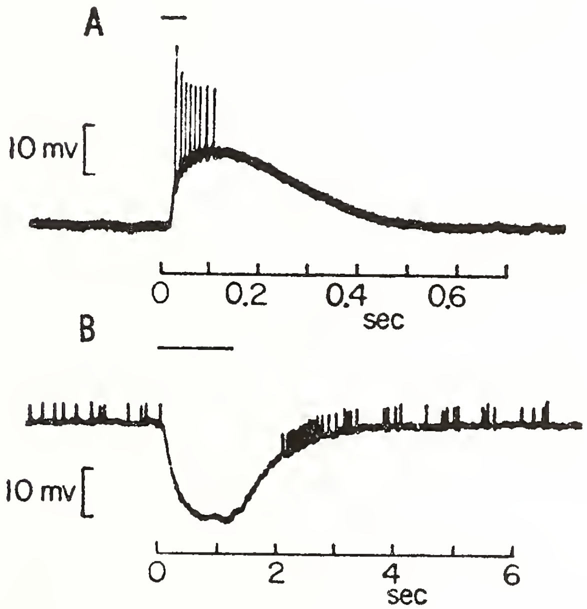

Figure 4. Receptor potentials with accompanying action potentials in excitatory proximal (A) and inhibitory distal (B) photoreceptors of *Argopecten (=Pecten) irradians.* Membrane depolarization exceeds threshold and triggers a spike burst in the proximal receptor (A). Hyperpolarization inhibits ongoing spontaneous activity in the distal receptor followed by a burst of spikes with depolarization following light off. The inhibitory response is similar to the inhibitory model (Fig. 2). From McReynolds and Gorman (1970a).

Receptor physiology has been studied in the eyes of only a single additional bivalve species, the giant clam *Tridacna maxima* (Wilkens 1988). Although no recordings were made from optic nerve fibers, responses to light recorded intracellularly from retinal cells are hyperpolarizing, consistent with primary inhibition characteristic of bivalve photorsensitivity. Unlike the scallop, however, two types of inhibitory photoreceptors have been described, and no excitatory cells were found. Classified as spiking (S) and non-spiking (NS) receptors (Fig. 5), these cells can be distinguished by the following criteria. The S-cells, which share most of the characteristics of scallop distal photoreceptors, are hyperpolarized by light and spikes are suppressed. Similarly, light has a secondary excitatory effect seen in the strength of the spike burst arising from the off-response depolarization. This is illustrated (Fig. 5A) where spikes are absent in the second off-response as a consequence of reduced light adaptation from the initial dark period, and where the number of spikes increases relative to increases in duration of the preceding period of illumination (Fig. 5B). Spiking in NS-cells has never been observed. It is possible that the absence of spikes is due to injury from electrode penetration, as has been reported commonly for both cell types in scallops (McReynolds and Gorman 1970a). However, S-cells retain their ability to conduct action potentials as long as cell penetrations are maintained, for periods up to an hour. Further, *T. maxima* receptors are invariably distinguished by differences in membrane potential and light adaptation. S-cell receptor

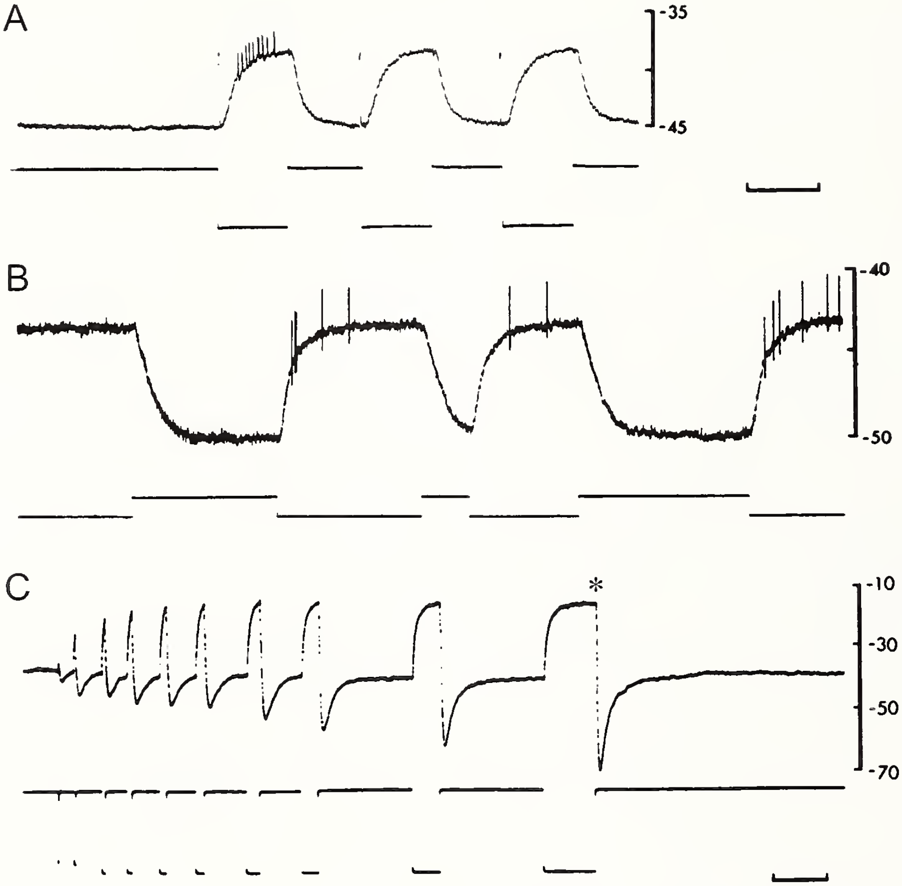

Figure 5. Receptor potential in *Tridacna* photoreceptors. Spiking S-cell off-response beginning when fully light adapted following several minutes under bright illumination (A) and beginning when fully dark adapted (B). The light-off burst of spikes is greatest for higher degrees of light adaptation, *e.g.*, the initial dark period in (A) and spike counts in response to relative durations of light exposure in (B). Non-spiking NS-cell (C) beginning in a fully light-adapted state followed by shadow stimuli of increasing duration. Inhibitory hyperpolarization in response to light increases following increasing intervals of illumination. Relative light adaptation in S-cells is evident in the spike burst response but not in receptor potential amplitude, whereas NS-cell responses change dramatically with relative light adaptation. The light stimulus is monitored in the lower trace of each pair; light on is the upward position. Vertical scale indicates actual membrane potential in millivolts; horizontal bar = 30 s in (A) and (C), 5 s in (B). Modified from Wilkens (1988).

potentials are seemingly immune to adaptation, either in the light or in the dark (Fig. 5A-B), whereas the receptor potentials of NS-cells respond variably as a function of light exposure (Fig. 5C). At the beginning of this record (Fig. 5C), the cell is fully light adapted and inhibitory-like hyperpolarizations increase dramatically following increasing durations in the dark. On-response hyperpolarizations also adapt rapidly, returning to the light-adapted baseline irrespective of changes in relative dark adaptation as seen throughout this record. Membrane and receptor potentials also vary consistently, with average resting (dark) potentials of −44 mV in S-cells and −14 mV in NS-cells and hyperpolarizing off-responses averaging 14 mV or up to 100 mV in fully light-adapted S- and NS-cells, respectively. Both cell types have axons that emerge from the receptor soma, as seen in injections of fluorescent dyes following recording sessions (Wilkens 1988). These converge to form the optic nerve described morphologically by Stasek (1966) and Kawaguti and Mabuchi (1969). Neither Kawaguti and Mabuchi (1969) nor Fankboner (1981) report synapses in their electron micrographs of the *T. maxima* retina.

Receptor physiology, consistent in its inhibitory response to light, is nevertheless significantly different in the eyes of scallops and giant clams. Differences, as yet unexplained, also exist in the organization of the visual apparatus. The dual retinas in scallops form a common optic nerve that projects without branching or synapse formation into the lateral (optic) lobes of the parietovisceral ganglion (Spagnolia and Wilkens 1983, fig. 6). Any mantle reactions to light are therefore dependent on efferent projections back to the periphery. In *Tridacna maxima* small pieces of mantle tissue, excised and pinned out for recording, exhibit local retractions to shadows. This requires that optic fibers synapse with other cells after leaving the eye capsule, or that dermal photosensitivity exists in the mantle aside from the eyes, as in *Lima scabra* (Mpitsos 1973). Receptor organization is also different, as histological studies in *T. maxima* do not show the distinct retinal layers characteristic of scallop eyes. *Tridacna maxima* receptor cells are described by Fankboner (1981) as having numerous ciliary processes that give rise to tangles of microvilli whereas Kawaguti and Mabuchi (1969) distinguish two cell types, one of which is described as rhabdomeric but having ciliary basal bodies. Both authors may be describing the same ciliary receptor somewhat differently, but no evidence as yet relates any structural difference to the physiologically distinct S- and NS-cells.

Contrasting ionic mechanisms of hyperpolarizing receptor potentials

The receptor potentials and mechanisms of phototransduction in scallops have been studied extensively with respect to their differing polarity. In *Argopecten irradians* ir-

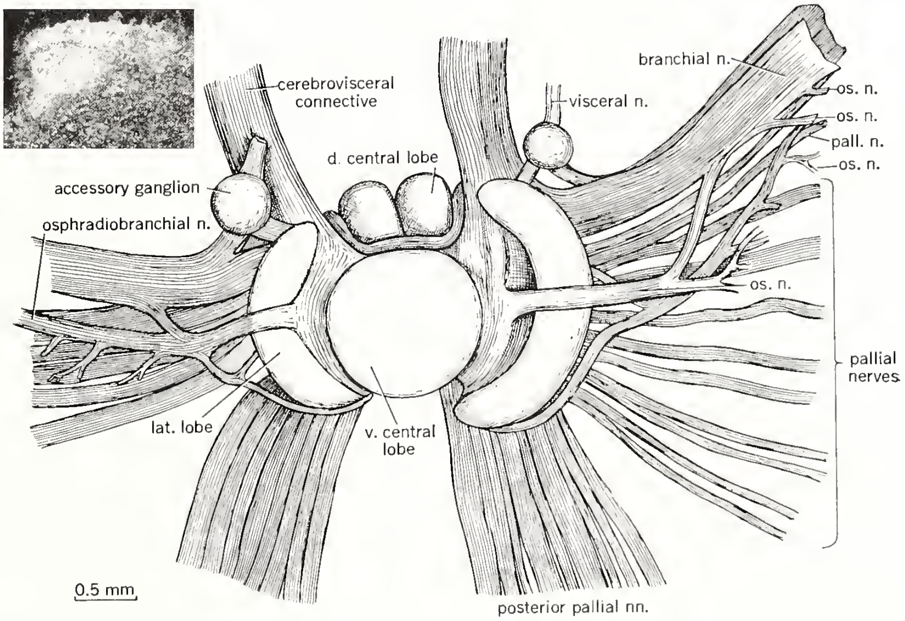

Figure 6. Diagram of the parietovisceral ganglion of *Pecten*. From Dakin (1910). The larger lateral lobe on the right corresponds with the upper (left) mantle that contains a larger number of eyes than present on the lower (right) mantle. The inset (Wilkens, unpublished) shows autoradiographic labeling of cortical neurons (surface layer) and a spherical glomerulus after injection of tritiated proline into the eye and axonal transport via the pallial nerves into the lateral lobe.

radians, both depolarizing and hyperpolarizing receptor potentials are based on increases in ionic conductance (McReynolds and Gorman 1970b). The excitatory depolarizing potentials of arthropods, and presumably other invertebrate photoreceptors, also arise from increases in membrane conductance (Fuortes 1959), whereas in vertebrate receptors the 'inhibitory' hyperpolarization is associated with a decrease in conductance (Toyoda *et al.* 1969). Therefore, receptor polarity and conductance changes must be explained as a function of ion channel specificity. In scallop proximal receptors, an increase in inward sodium conductance drives the excitatory depolarization (Gomez and Nasi 1996), similar to other depolarizing receptors. In the distal receptors of scallops, which have received the greatest attention, hyperpolarization is due to an increase in outward potassium conductance (Gorman and McReynolds 1978), with similar ion specificity in *Tridacna maxima* (Wilkens 1988). These experiments are based on ion substitution and reversal potential measurements, with similar results in scallops and giant clams. However, hyperpolarizing light inhibition, as seen in bivalve photoreceptors and in vertebrate rods and cones, is based on contrasting mechanisms. In bivalves, hyperpolarization is due to an *increase* in conductance, *i.e.*, outward potassium current. In vertebrates, hyperpolarization is due to a *decrease* in conductance shutting off the leaky, inward sodium currents that depolarize the cell in the dark. Thus, the inhibitory effects of light are explained ultimately by ion channel specificity and its activation or suppression by the photochemical mechanisms following light absorption. For the scallop distal photoreceptors, as in vertebrates and other animals, much additional work has been directed towards understanding the cascade of second-messenger intermediates (Gomez and Nasi 2000), the evolutionary history of opsin photopigments, and their control of membrane conductance, and bivalve photoreceptors remain of great value in the comparative study of phototransduction.

Historically, the evolution of the eye has been of immense interest, including the morphology of its light-sensitive cells. Photoreceptors generally involve an extensive membrane hypertrophy involving either the cilium or microvilli of the cell membrane in the formation of a rhabdom. Aside from the competing theories concerning the phylogenetic relationships of photoreceptor morphology, some trends are in evidence physiologically. For example, all depolarizing/excitatory photoreceptors are exclusively rhabdomeric, including the examples mentioned previously, *e.g.*, all arthropods, annelids, gastropods, cephalopods, and the proximal receptors in pectinid eyes. In contrast, hyperpolarizing photoreceptors are predominantly ciliary although the list is fairly short, *e.g.*, vertebrate rods and cones, the distal photoreceptors and siphon/mantle nerves in bivalves, and the photoreceptors in the larval eyes of the tunicate *Amaroucium constellatum* (Gorman *et al.* 1971). A lone exception is found in the eye of another tunicate, *Salpa democratica*, where the hyperpolarizing response is associated with a microvillar-type receptor although these eyes are considered non-homologous to those of other primitive chordates (Gorman *et al.* 1971). In another exceptional instance, a putative ciliary photoreceptor is found in the brain of the polychaete annelid *Platynereis dumerilii* (Arendt *et al.* 2004) although its response to light is unknown. Ciliary opsin similar to that of vertebrate rods and cones is associated with these photoreceptors but not the rhabdomeric receptors of the eyes.

Despite these trends, information is insufficient for linking physiological sensitivity to the evolution of photoreceptor morphology. Hyperpolarization is seemingly a characteristic of all chordate photoreceptors, as it is in bivalve eyes. However, the ionic and conductance mechanisms are distinctly different between these two groups. In addition, the depolarizing excitatory receptors of scallops are anomalous considering the bivalve emphasis on primary inhibition. Genetic analysis of the various phototransduction mechanisms is the likely avenue for resolving the evolutionary relationship of photoreception among animals. Nevertheless, further sampling of the physiological properties of bivalve eyes would be of great utility in understanding visual function in this sedentary group, including both cephalic eyes in the Arcoida and Pterioida and the rich diversity of pallial eyes, as reviewed by Morton (2001).

Behavioral implications from receptor physiology

The evolution of an inhibitory light response restricted to just two groups of unrelated animals makes for interesting comparisons. Inhibition in vertebrates must be viewed in the context of a complex retina with an extensive set of integrative components underlying image formation and dependent on the functional specificity of synaptic contacts from rods and cones to their post-synaptic targets. Transmitter release from receptors in a depolarized state is high while in the dark. With hyperpolarization transmitter release is reduced. However, the 'inhibitory' light response can be interpreted as either inhibitory or excitatory postsynaptically, depending on the specificity of ion channel activation by the neurotransmitter. For example, light inhibition that suppresses the release of an inhibitory transmitter will result in excitation while suppression of an excitatory neurotransmitter will have a net inhibitory effect. These and other synaptic interactions are integral to the construction of receptive fields in elements of the retina that are essential for spatial imagery, along with focus of the lens.

In bivalve vision, inhibition by light is presented directly to the central nervous system as an inhibitory signal, *i.e.*, the cessation of activity from optic (or pallial) fibers that would otherwise be excitatory. However, it is generally accepted that a photoreceptor inhibited by light at a low-threshold, but also primed by a latent light-mediated excitation, is physiologically efficient for responding to a shadow (Land 1966), where spikes are triggered with minimal delay on the rising phase of the receptor potential (McReynolds and Gorman 1970a). Thus, bivalves have encoded response properties optimized for detecting shadows directly into their sensory receptors. In doing so, they avoid delay intervals inherent in the synaptic activation of second-order neurons for mediating an off-response, as is the case for the shadow response in barnacles where the effect of light is excitatory at the receptor level (Gwilliam 1963).

Light inhibition and the ensuing off-response in distal receptors is also optimal and potentially most important for signaling motion sensitivity. The distal retina lies in the image plane of the mirror-like optics of the scallop eye (Land 1965) and the off-response receptors are therefore shadowed or illuminated sequentially by movements in the visual field. A moving object whether light or dark is an effective stimulus since movement will invariably darken some part of the retina (Land 1966). The excitatory receptors in the proximal retina lie outside the image plane, lack spatial acuity, and also adapt rapidly to light (McReynolds and Gorman 1970a) and under lighted conditions would be less responsive to rapid increases or decreases in light intensity. Thus, the more phasic off-receptors inhibited by light are optimally designed and positioned in scallops to detect movement, signals that represent potential predators for a sedentary animal, swimming not withstanding. Off-receptors perform the same function in the giant clam although without the benefit of an image-forming mirror in what Stasek (1965) refers to as the 'sight reaction'. Movements in the environment trigger rapid mantle retraction and valve adduction without shadowing to avoid the potential grazing of predatory reef fish. Spatial resolution is limited to that provided by the aperture of the invaginated pinhole eye, but Land (2003) has nevertheless measured acceptance angles for individual receptors that correspond to the motion-induced behaviors. Primary inhibition in bivalves can therefore be viewed as an evolutionary adaptation for survival, whether for responding to a shadow by animals lacking eyes or responding to movement in the environment. Movement detection has been likened to the function of a burglar alarm (Nilsson 1994), giving the animal advance warning not available to animals lacking eyes and dependent on shadows.

Nevertheless, many aspects of bivalve vision remain poorly understood, including whether or not eyes are essential given that the vast majority of species have no eyes (Morton 2001). At the opposite extreme, the ark clams have hundreds of eyes. Also, there is great diversity in eye morphology among the species where eyes do exist (Nilsson 1994, Morton 2001) and physiological information is lacking except for the handful of species discussed here. Indeed, it is an open question whether eye diversity is homologous (Nilsson 1994) so receptor physiology could provide additional insights concerning the evolution of bivalve vision.

Aside from the burglar alarm theory, what function do eyes provide? In addition, what is the function of the depolarizing on-receptors found so far only in scallops and the file clam? Despite the evidence for image formation in scallops there is little indication that images are useful, other than for motion sensitivity, and doubt exists as to whether the central nervous system has the computational machinery required to process an image (Morton 2001).

The functional morphology of eyed bivalves still invites inquiry into the question of spatial vision. In scallops, for example, optic tracts entering the parietovisceral ganglion via the pallial nerves innervate the lateral lobes, the functional equivalent of 'optic' lobes (Fig. 6). Optic fibers make initial synaptic contact with a cortical layer of second-order neurons that in turn communicate with glomerular structures deeper into the optic lobes (Spagnolia and Wilkens 1983). Simple methylene blue staining of live tissue shows that the lateral lobes contain numbers of glomeruli in proportion to the eyes in the mantle, and in the diagram of the *Pecten maximus* ganglion (Fig. 6), the larger size of the left lobe reflects the greater number of eyes on the corresponding dorsal mantle. Glomerular structures are characteristic of high-level sensory integration so it is tempting to speculate that these structures make use of neural images from the eye. Curiously, however, in recordings from the cortical surface of the lateral lobes in *Euvola (=Pecten) ziczac* (Linnaeus, 1758), light stimuli elicit far greater activity from second-order visual neurons associated with on-responses in the optic nerves than for off-responses (Wilkens and Ache 1977). This suggests a high degree of input from the depolarizing proximal receptors although their 'unfocused' role has been suggested as being limited to detecting only changes in overall light intensity. New evidence (Speiser and Johnsen 2008) that both retinal layers receive focused images from the optical mirror is consistent with the predominance of excitatory input centrally. Visual function in scallops remains as an interesting avenue for further research.

Vision, when present in bivalves, involves numerous eyes distributed around the mantle/shell margins of the animal with numbers ranging up to hundreds in arcaceans. In the ark clams, pigment-cup and compound eyes are present, both in high numbers but with poor resolution (Nilsson 1994). The large number of eyes and even larger number of ommatidia are well designed to function as an alarm system with a high safety factor estimated at up to 755 receptor units for any direction in the visual field (Nilsson 1994). The visual system of *Tridacna* is also comprised of a large number of eyes, and with a high degree of overlap considering the scalloped arrangement of the mantle lobes and radial orientation of the eyes plus an acceptance angle for individual receptors estimated at 16.5° (Land 2003). In essence, all of these visual systems have the potential to form a mosaic type of image projecting to the central nervous system. Even animals without eyes nevertheless are able to form a diffuse spatial image, as demonstrated in echinoids based on their orientation and movement toward large contrasting objects (Blevins and Johnsen 2004). Scallops have functional optic lobes, but very little is known for other bivalves about visual integration or the functional morphology for vision in the central ganglia. Visual behavior in giant clams suggests at least the possibility that a coarse visual image of the environment can be constructed. Stasek (1965) notes anecdotally that *Tridacna* has been observed to aim the exhalent cone at objects held over the mantle, much as they do when spurting water in the direction of mantle irritants, *e.g.*, a grazing reef fish. While this behavior has not been confirmed, valve adduction and mantle retraction responses in these clams have been shown to habituate to a moving shadow presented repetitively in one part of the visual field but remaining responsive to a novel motion stimulus in another area (Wilkens 1986). In one instance, a giant clam habituated in this fashion responded vigorously to the shadow of a passing cloud. These behaviors suggest that some degree of discrimination for objects or movement in the visual field exists and that this capacity requires a type of collective image formation centrally. It remains that bivalves, as a largely sedentary group of animals, have at best limited needs for a sensory system with high visual acuity. Nevertheless, the 'evolutionary energy' in developing functional eyes and a behavioral repertoire in a few species challenges us to explain the adaptive significance of these visual systems. This is especially true for the scallop and file clam where both inhibitory and excitatory photoreceptors are present and the animals are capable of locomotory responses.

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When a snail dies in the forest, how long will the shell persist? Effect of dissolution and micro-bioerosion

Timothy A. Pearce

Carnegie Museum of Natural History, 4400 Forbes Ave, Pittsburgh, Pennsylvania 15213, U.S.A., PearceT@CarnegieMNH.org

Abstract: Snail shells persist in the environment after death, but we know little about the rate at which shells decompose. Assumptions about the rate of shell decomposition are relevant to conservation biologists who find empty shells or biologists using empty shells to make inferences about assemblages of living individuals. I put shells in 1.6 mm mesh litter bags (excluding macro-grazers) in Delaware and northern Michigan, U.S.A. and monitored shell mass annually for 7 years. Decomposition rates differed among species, but I found no difference in rates at two sites with different habitats. Surprisingly, loss of periostracum had no effect on shell decomposition rate. At the locations and habitats studied, decomposition rate of snails averaged 6.4% per year, excluding shells that broke during the experiment (shell half life = 11.5 years), or 10.2%, including shell breakage (half life = 7.5 years). Half lives would likely be shorter if macro-grazers had access to shells. These results caution us to draw conclusions carefully when including empty shells in inferences about assemblages of living individuals.

Key words: chemical weathering, death assemblage, decomposition rate, land snail, periostracum

After snails die, their shells persist in the environment. Although some shells survive as fossils for hundreds of millions of years, most shells decompose (or effectively disappear) more quickly than that, probably on the order of months or years. A shell in a dry, protected place such as a desert or a museum might persist for hundreds of years. A shell on the forest floor might persist more briefly—but how briefly?

Although we know little about the decomposition rates of land snail shells in leaf litter, many studies make assumptions about the rate of shell decomposition and could benefit from more information about the decomposition rates (Menez 2002). Management biologists making conservation decisions would find decomposition rates relevant for knowing how long ago a species was living at a site where an empty shell was found. Although using data from snails collected alive would give more reliable results, biologists conducting biodiversity surveys commonly use empty shells as an expedient way to indicate the presence of species at a site. Furthermore, since methods for recovering snails from leaf litter (*e.g.*, sieving and picking snails) are labor intensive, including information from empty shells is tempting for at least two reasons. First, empty shells can usually be recovered along with live specimens with little extra effort, and second, the only occurrence of rare species in a sample might be empty shells, so excluding empty shells would discard information. Studies in which empty shells and live shells are counted indiscriminately would, of course, overestimate population sizes of the living snails. Furthermore, if shell decomposition rates differ among species, then including dead shells would overestimate the abundance of robust-shelled species. Using empty shells to calculate proportions of species in the assemblage of living individuals requires assuming that the death assemblage accurately represents the assemblage of living individuals. This assumption might be incorrect if different species, robust and fragile-shelled, decompose at different rates or if shells at different sites decompose at different rates.

Shells of *Ovachlamys fulgens* (Gude, 1900) decomposed in an average of five months in Costa Rica during the dry season (Barrientos 2000). Aside from that study, most of what we know about the rate at which snail shells decompose is anecdotal. Welter-Schultes (2000) collected all the dead shells of *Albinaria jaeckeli* Wiese, 1989 that he could find in a particular area once in 1987 and again in 1990. The number of dead shells he collected was similar in each year, suggesting that the dead shells had been completely replaced within three years.

Shells probably disappear by three main processes: dissolution, breaking, and bioerosion (shell removal by grazing). Shells that are protected from bioerosion decompose more slowly than shells that are exposed to this process (Cadée 1999). Although consumption by larger organisms such as other snails and decomposition by crushing and breaking are real processes contributing to disappearance of shells, in this study I excluded macro-grazers larger than 1.6 mm and breakage (for most analyses) by keeping target snails in mesh bags. Consequently, the shell decomposition rates in this study are likely to be slower than in experiments allowing access by macro-grazers such as other snails. However, excluding macro-grazers allowed me to focus on the effects of dissolution and micro-grazers less than 1.6 mm that might remove shell material by grazing.

In this study, I address 4 questions: (1) Do shells of different species decompose at different rates, (2) Do shells in different habitat types decompose at different rates, (3) Does periostracum loss influence shell decomposition rate, and (4) What is the mean half life of a dead snail's shell?

MATERIALS AND METHODS

Localities and species

To address how long empty shells persist in the forest, and to test for differences in decomposition rates among species and among sites, I put individually numbered shells in mesh litter bags at sites of two different habitats in northern Michigan and at one site in Delaware with three replicates located about 15 meters apart at each locality. The litter bags were approx. 22 × 22 cm with 1.6 mm screen openings and held about 1 liter of soil, leaf litter, and the decomposing shells. The litter bags were placed on the mineral soil surface and covered with about 5 cm of leaf litter and a small amount of soil. I monitored shell mass annually for 7 years.

Measuring mass loss from material in mesh bags has been used in many studies of leaf litter decomposition (Taylor and Parkinson 1988) and is an applicable method to studying snail shell decomposition. Choice of mesh size in the bags is a trade-off between retaining fragments and allowing entrance by grazing organisms. If larger animals were important grazers on shells, then mesh sizes that exclude them will result in slower shell decomposition rates. For example, such a decrease in decomposition rate was observed in studies of leaf litter between larger mesh that admitted and smaller that excluded grazers (Cornelissen 1996).

The two northern Michigan localities included a mixed pine and hardwood forest on a sandy outwash plain and a mixed hardwood forest on rich moraine soil. At these localities, I used locally collected shells from near the sites where I studied their decomposition. The Michigan outwash plain site tends to be drier because sandy soil does not hold water as well. In Delaware, I used shells collected from the Delmarva Peninsula and put them in a beech-maple forest on piedmont. Soil pH measured 4.5 at all sites.

In Michigan, I used shells of 7 species: *Anguispira alternata* (Say, 1816) ($n = 48$), *Discus catskillensis* (Pilsbry, 1896) ($n = 18$), *Euchemotrema fraternum* (Say, 1824) ($n = 8$), *Haplotrema concavum* (Say, 1821) ($n = 18$), *Mesodon thyroidus* (Say, 1816) ($n = 18$), *Neohelix albolabris* (Say, 1817) ($n = 6$), and *Novisuccinea ovalis* (Say, 1817) ($n = 2$). I chose shells that ranged in size from 4 to 25 mm diameter and ranged from the relatively robust and thick-shelled *A. alternata* to the thin and fragile *N. ovalis*. I individually numbered shells with India ink and divided specimens of each species evenly into the 6 replicate bags (3 from each habitat), for example, 8 *A. alternata* in each bag. For species that did not divide evenly by 6 (*e.g.*, *N. ovalis* and *E. fraternum*), I put one remainder in each of the outwash plain and the moraine localities. The Delaware bags each contained 7 *Triodopsis fallax* (Say, 1825).

At the start of the experiment, shells ranged from fresh to eroded and some had been broken by small mammal depredation. The fact that some shells were not fresh at the start is not a problem because I compared relative shell loss from year to year. Although older shells might be expected to decompose more rapidly, for example due to periostracum loss, as will be seen in the results, periostracum loss had no significant effect on percentage annual shell mass loss. The Delaware shells were intact, but most were missing some periostracum at the start of the experiment. In Delaware, I used only one species, *Triodopsis fallax*, which has a fairly robust shell.

The fact that the mesh bags in Michigan and Delaware had no species in common means that I cannot examine species-locality effects among all three localities. However, since I used the same species at both Michigan sites, I can look for species-locality effects there. Because sample sizes of some species were very small, caution should be exercised in interpreting results.

Analyses

To determine shell decomposition, I weighed shells annually after retrieving them from the litter bags, cleaning off adhering soil, and air-drying them to constant mass. Cleaning and drying resulted in little to no observable shell loss although a few small non-adhering pieces of periostracum occasionally fell off shells during drying. I did not retrieve shell fragments less than about 4 mm^2, so shells with these kinds of small fragment losses are interpreted in this study as shell mass loss through decomposition. Larger shell fragments that could be associated with an individual shell were included in the mass measurements of that individual. In addition, in order to examine mass loss by shell breakage and to examine the effect of periostracum loss on decomposition rate, I also annually estimated the proportion of the shell and periostracum that were missing and measured maximum diameter of the remaining shell. I re-inked identification numbers onto the shells if the previous numbers had faded.

I made some adjustments to the data set. In some instances, the apparent mass of a shell increased over a previous year (perhaps a piece of sand had lodged in the shell). For instances in which the mass of a shell increased more than 10%, I removed the increased year from the analysis.

I excluded the first year of *Discus catskillensis* measurements from the analyses. The shells had been collected alive and dried without removing the bodies. The shells lost much more mass the first year (mean of 5 mg or 46% of shell mass) compared to subsequent years (0.5 mg or 15% of shell mass), suggesting that the soft tissue body masses had been a significant part of the mass the first year. Indeed, Pearce and Gaertner (1996) reported dry mass of *D. catskillensis* to be about 2 mg. Because soft part decomposition the first year seemed to account for a large portion of the mass loss, I excluded all first-year *D. catskillensis* from mass loss analysis.

In most analyses, I was interested in the shell mass loss due to non-breakage factors rather than shell loss due to breakage. To focus on effects of dissolution and micro-grazers, I excluded from statistical analyses shells that suffered catastrophic breakage during a particular year. I included shells that were intact for at least 2 contiguous years. I defined broken shells as those that lost more than 15% of shell (estimated visually and recorded annually) or more than 5% shell maximum dimension (measured and recorded annually). Defining shell breakage using the percent shell present was independent of any changes in shell mass.

Although a control was not used in this experiment, for example, to assess repeatability of measurements from year to year, the precision of measurement obtained was much greater than the variation from year to year.

Statistical tests and comparisons

The primary measure I used to assess shell decomposition was decrease in mass over time. In order to standardize so shells of different starting masses could be compared, I calculated % shell mass loss over time and used this measure in comparisons. I used this measure for addressing questions comparing shell decomposition rates among different species and different localities. A test for normality of percent shell mass loss of *Anguispira alternata* showed the data to be kurtotic; sample sizes of the other species were too small to allow tests for normality. Consequently, I transformed the data using Log(x+1) and used ANOVA to compare different species or localities. I used the Tukey test to examine post-hoc differences.

In order to evaluate whether shell decomposition rate increased after periostracum loss, I examined whether shell mass loss rate correlated with percent periostracum loss.

To calculate the half life of the shell, I extrapolated the shell decomposition rate to determine when half the mass would remain.

RESULTS

Examples of shells that had decomposed for 4 and 7 years are shown (Fig. 1). An example demonstrating how the mass changed for individual shells of *A. alternata*, for 3 shells that remained intact, and 3 shells that experienced catastrophic breakage at some point in the 7 year experiment, is shown (Fig. 2).

Shell decomposition rate differed among species in 119 shell specimens that did not break (ANOVA, $F = 3.774$, $P = 0.001$) (Fig. 3). The Tukey post hoc test showed that decomposition rate of intact *Anguispira alternata* was less than that of *D. catskillensis*, and *M. thyroidus* was less than those of *D. catskillensis, H. concavum,* and *T. fallax*. Interestingly, larger shells had a slower percentage shell mass loss rate than smaller shells (Pearson correlation, $N = 142$, $R^2 = 0.088$, $P < 0.001$, unbroken shells only, not shown). Of the 5 species having at least 10 unbroken specimens, *A. alternata* was the only one showing a significant within-species correlation of shell mass loss with shell size (Pearson correlation, $N = 48$, $R^2 = 0.099$, $P < 0.05$), suggesting that it might be the major contributor to the correlation for all species, although its pattern is not contradicted by the trends in other species. When I subjectively classified *N. ovalis* and *H. concavum* as relatively fragile shells and the rest as relatively robust shells, I saw no striking difference in trends for percent shell mass loss.

Shell decomposition rate did not differ significantly between the moraine site and the outwash plain site for 100 unbroken specimens in Michigan (ANOVA, $F = 2.536$, $P = 0.114$). Because different species decompose at different rates, and the shells in Delaware were different species from those in Michigan, if there were differences between Michigan and Delaware specimens, I would not be able to differentiate species differences and locality differences. Consequently, I omitted Delaware from the analysis comparing shell decomposition rates among localities.

Surprisingly, shells that lost more periostracum did not decompose faster (Pearson correlation, $R^2 = 0.0004$, $P > 0.5$) (Fig. 4). Although all values of mass loss per year greater than 22% had less than 11% periostracum remaining, that apparent greater variability likely reflects the larger statistical sample of shells with little or no periostracum remaining. Furthermore, five species having sample sizes of at least 12 individuals had shell decomposition rates independent of periostracum loss (separate species P-values > 0.2 to > 0.5). Although periostracum loss itself did not affect shell decomposition rate, it varied among species in 113 shells examined (ANOVA, $F = 4.997$, $P<0.0005$) (Fig. 5).

Considering the intact shells only, which decomposed at an average of 6.4% per year, the half life of an individual shell (protected from macro-grazers) would be 11.5 years and after 35.8 years only 10% of the shell would remain. Considering both intact and broken shells, which decomposed at an average of 10.2% per year (Fig. 3), the half life

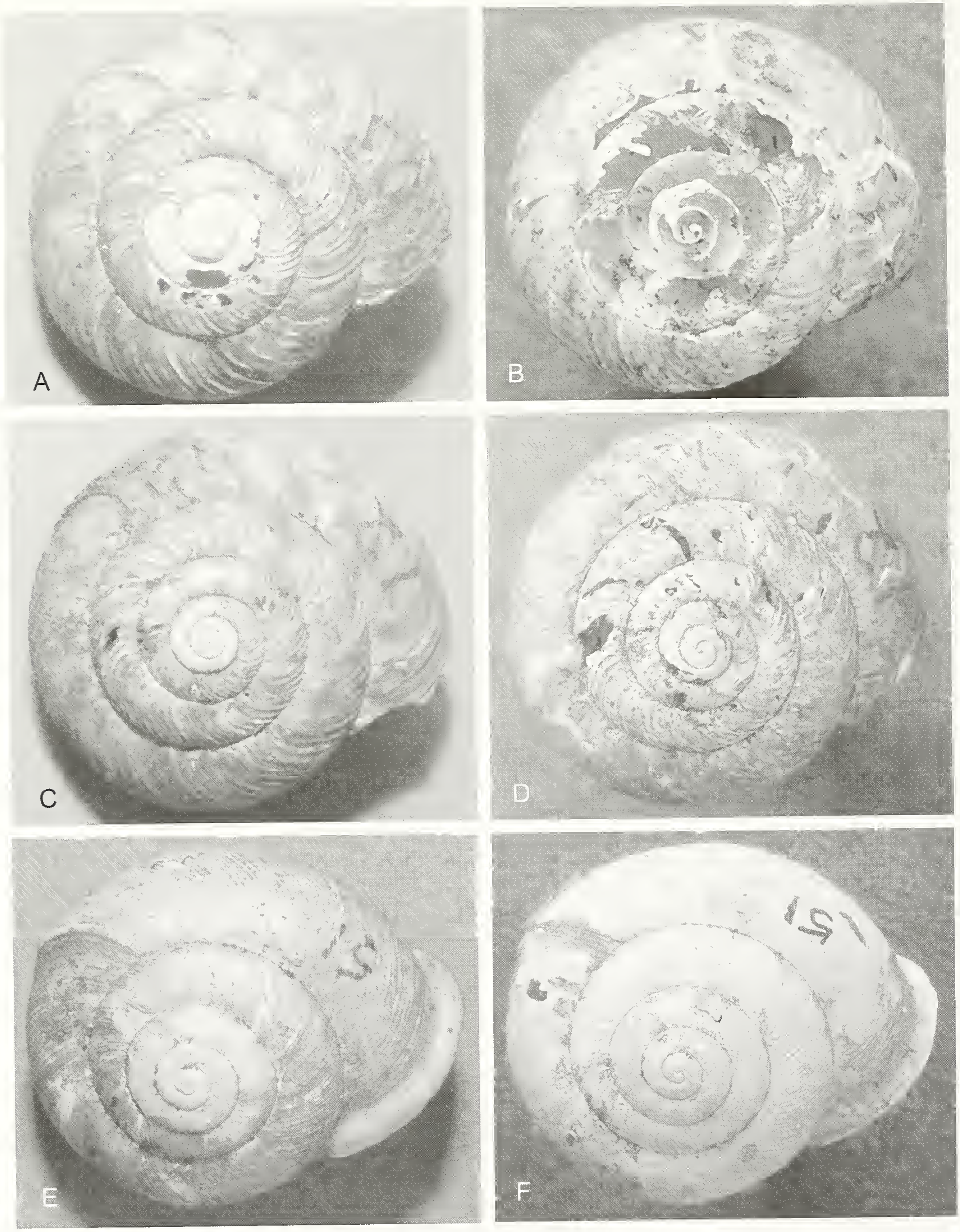

Figure 1. Appearances of three individual shells after 4 years (left column, A, C, E) and 7 years (right column, B, D, F): *Anguispira alternata* No. 03 (13.6 mm diameter; A, B), *A. alternata* No. 12 (17.6 mm; C, D), *Mesodon thyroidus* No. 51 (23.1 mm; E, F).

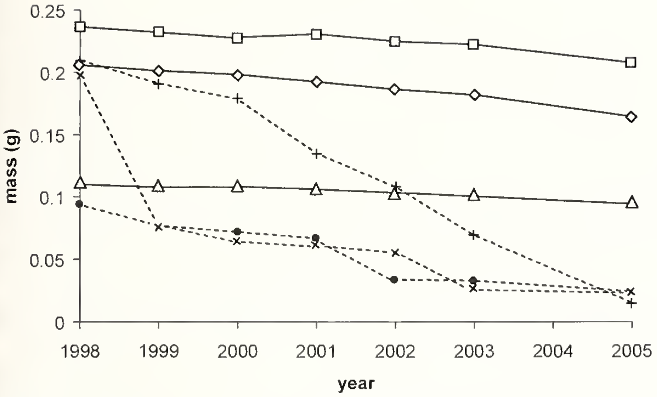

Figure 2. Loss of *Anguispira alternata* shell mass over 7 years. Dashed lines are shells that broke during the experiment; solid lines are shells that remained intact.

of the shell would be 7.5 years; after 22.4 years, only 10% of the shell would remain.

DISCUSSION

Because shells of different land snail species decompose at different rates, these results demonstrate that using shells from dead snails has potential to bias estimates about assemblages of living snails. Admittedly, sample sizes of some species in this study were very small, so results about those species must be interpreted with caution; however, being cautious with those results does not change conclusions about species with larger sample sizes. Conclusions in studies using empty shells should indeed be drawn carefully.

Shell decomposition rates are likely influenced by a plethora of factors. Three of the factors that might influence shell decomposition rates are surface area to mass ratio, shell robustness, and physical and chemical environment. Larger shells, which likely have a smaller surface area to mass ratio, lost mass more slowly than smaller shells in this study. Menez (2002) also found that larger snail shells degraded more slowly. Such a size difference might be expected since shells with a high surface area to mass dissolve more rapidly (Claassen 1998). Although physical and chemical destruction has been reported to be faster in thin-shelled than more robust species (Evans 1972), no effect of shell robustness was observed on shell decomposition rate in this study although a better test for an effect of robustness needs to be conducted. Robustness would be influenced by shell thickness as well as form, such as ridges that add strength; future tests of robustness should examine crush strength among species.

Shell decomposition rates did not differ significantly at two habitats in Michigan, despite the habitats differing in substrate (sandy soil versus poorly sorted moraine deposits), vegetation (relatively low oak and pine with sparser undergrowth versus taller aspen forest with denser undergrowth), and evidently moisture (although the pH did not differ). This result contrasts with leaf litter decomposition rates, which are slower in sandy soil having less moisture and less nutrient-holding capacity (Johnson *et al.* 2000), suggesting that processes regulating leaf litter and shell decomposition might differ. Because temperature, moisture, and pH likely play important roles in shell decomposition, shells in environments different from those I studied are likely to have different decomposition rates. Indeed, Barrientos (2000) found that shells in mesh bags (mesh size not stated) in Costa Rica decomposed in an average of 5 months.

Surprisingly, periostracum did not seem to play a protective role in decomposing shells. Periostracum is usu-

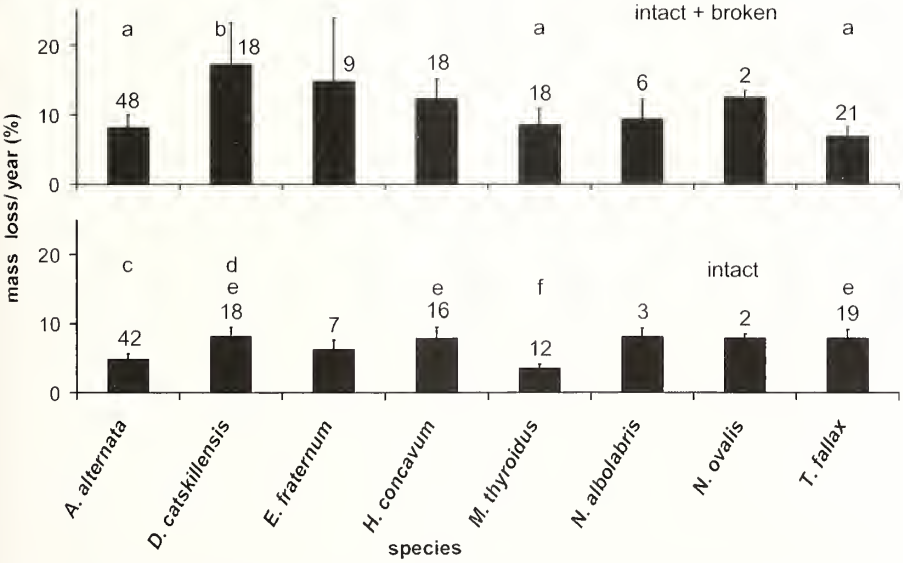

Figure 3. Shell decomposition rate contrasted among species. Lower panel indicates decomposition rate for unbroken shells, upper panel the rate for both broken and unbroken shells together. Numbers above bars indicate initial sample size. Bars with different letters within a horizontal row differed significantly; those without letters did not differ significantly. Data from broken shells were included in the lower graph only for their unbroken duration, which explains unequal mass losses in top and bottom graphs for species having same sample sizes.

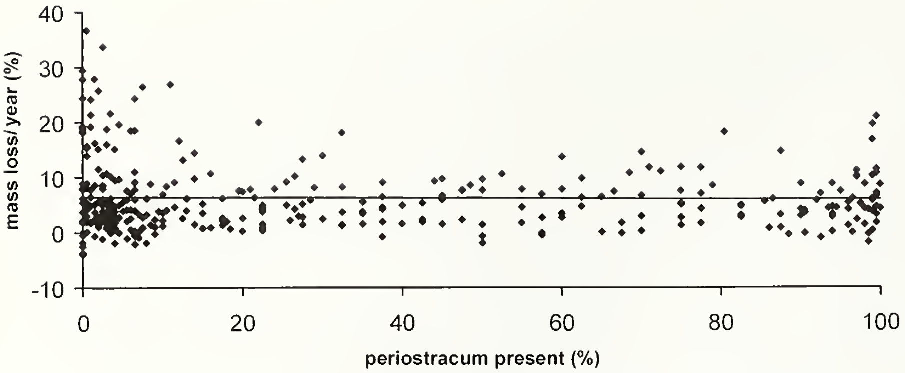

Figure 4. Shells that lost more periostracum did not decompose faster. Points show amount of periostracum remaining at the end of a year (x-axis) and amount of weight loss since the preceding year (y-axis). Individual shells can appear more than once for different years.

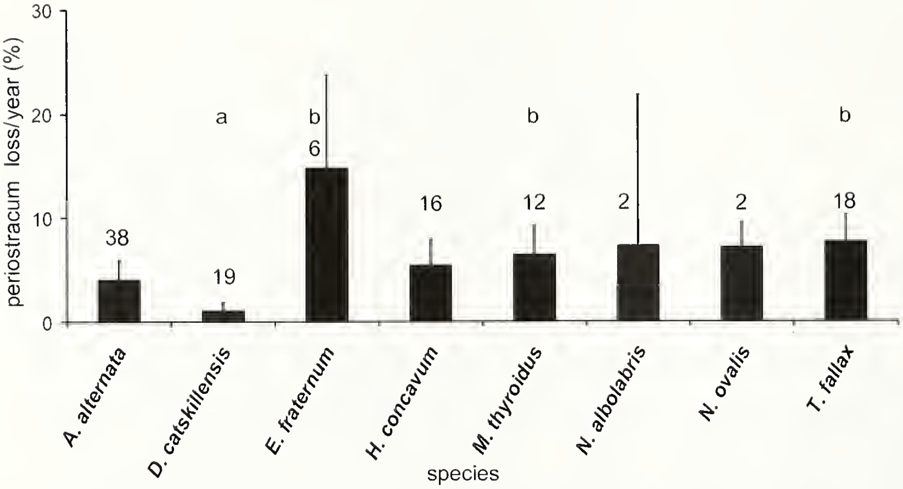

Figure 5. Periostracum loss rate varied among species. Numbers above bars indicate sample size. Bars with different letters within a horizontal row of letters differed significantly; those without letters did not differ significantly.

ally thought to protect shells from boring organisms, erosion, or dissolution by leaf litter acids in terrestrial snails or acidic water in aquatic molluscs (Solem 1974). While most living shells have intact periostracum, the apices of some living shells do erode over time. However, older molluscs missing large areas of periostracum do not seem to suffer serious erosion of the shell, suggesting that water and corrosion proofing qualities of the periostracum may be only of secondary importance (Hunt and Oates 1978). Possibly erosion soon after death starts on the inside surface of the shell, which is not protected by periostracum. Nevertheless, two questions remain: in the present study, why did the shell decomposition rate not increase after the loss of the periostracum, and why does periostracum apparently become more pervious after death, after having stayed intact for years during the snail's life? Regarding the second question, the living snail might behaviorally or chemically maintain a good bond between the periostracum and the shell whereas the bond might weaken after death, allowing ingress of corrosive solutions.

Living snails would be important grazers on decomposing snail shells. Other micro- or meso-organisms that might graze on decomposing shells are likely to exist. In a study of shell mass loss of *Helix aspersa* Müller, 1774 on a dune area in the Netherlands, Cadée (1999) found that shells protected from bioerosion lost about 8% mass in a year, similar to the rate found in this study. However, in that study, shells exposed to bioerosion by other land snails lost mass much more rapidly, 34% in 70 days, indicating that shells exposed to bioerosion could disappear in less than one year. If micrograzers are important contributors to shell decomposition, then the soil conditions (*e.g.*, nutrients) would also be relevant through their effect on the micro-grazers.

Dissolution and chemical conversion are often the main contributors to land snail shell decomposition (Claassen 1998). Colder water can dissolve more calcium carbonate (Claassen 1998) although more rapid dissolution can be expected at higher temperatures, at least in non-saturated water. On one hand, presence of moisture and lower pH increase the speed of shell decomposition (Claassen 1998, Reitz and Wing 1999). On the other hand, alkaline soils rich in calcium retard the breakdown of empty shells (Claassen 1998, Schilthuizen and Rutjes 2001, Cameron *et al.* 2003).

Although influences on decomposition rates of snail shells on forest floors are poorly known, insights might be gained from the more numerous studies on decomposition of leaf litter. Although different processes probably act on shells and leaves, results from studies finding leaf litter decomposition differences among habitats and climates are probably applicable to snail shell decomposition. For example, warmer climates would probably increase decomposition rates of shells, as it does in leaf litter (Bell 1974). In leaves, factors most important at determining the rate of decomposition are those that regulate microorganism activity: temperature, moisture, nutrients, and energy source (Berg and Ekbohm 1991). If microorganism activity plays a large role in shell decomposition, then shell decomposition rates would also be largely affected by processes regulating microorganism activity.

The result that snail shells of different species have different decomposition rates has important ramifications for studies of endangered species and community analysis. Finding empty shells of an endangered species in habitats similar to those studied here would suggest that the species was living in the area within the last several decades at most. However, the results of this study suggest that for community analysis studies, using empty shells to infer abundances of the assemblage of living individuals might violate the assumption that the death assemblage accurately represents the assemblage of living individuals. Including empty shells could overestimate the abundance of robust species.

In the geographical locations and habitats I studied, and with shells protected from macro-grazers, I extrapolate that shells will decompose to 10% of their former mass after several decades. For practical purposes, *e.g.*, in surveys recovering shells from leaf litter samples, shells missing more than 50% of their former mass might not be findable or identifiable, so shells in these conditions might effectively disappear in 7-12 years, their half life. Half lives would likely be shorter if macro-grazers had access to shells.

Future studies might help tease apart the processes involved in shell decomposition. Exploring the decomposition rates of shells in different environments (and geographic localities) and noting biotic and abiotic influences would help to address the importance of different situations (as has been found in leaf litter decomposition studies) and of scrapers or chemical weathering. Laboratory experiments could more directly evaluate the relative importance of the three decomposition methods and the importance of pH and temperature.

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Bivalve molluscs from the continental shelf of Jalisco and Colima, Mexican Central Pacific

Eduardo Ríos-Jara, Ernesto López-Uriarte, and Cristian M. Galván-Villa

Laboratorio de Ecosistemas Marinos y Acuicultura, Departamento de Ecología, Centro Universitario de Ciencias Biológicas y Agropecuarias, Universidad de Guadalajara, Carretera a Nogales km 15.5, Zapopan 40110, Jalisco, México, edurios@maiz.cucba.udg.mx, ernlopez@maiz.cucba.udg.mx

Abstract: A survey for bivalves was conducted at 25 sampling stations on the Mexican Central Pacific shelf off Jalisco and Colima, during the summer of 1988. The bivalves were sampled with a Van Veen grab at 16 stations with medium sand, sandy silt, and silty clay substrata at depths between 18 and 112 m. A total of 5,196 individuals belonging to 59 genera and 95 species of bivalves were found. A systematic list is provided with the relative abundance and density (individuals/m^2) for each species and information on depth, type of substratum, bottom water temperature, and oxygen concentration for each station. The twelve most common species (>100 individuals/station) in descending order of abundance were: *Nuculana laeviradius* (Pilsbry and Lowe, 1932), *Crassinella pacifica* (C. B. Adams, 1852), *Corbula nasuta* G. B. Sowerby I, 1833, *Anadara adamsi* Olsson, 1961, *Parvilucina approximata* (Dall, 1901), *Nucula declivis* Hinds, 1843, *Corbula ira* Dall, 1908, *Radiolucina cancellaris* (Philippi, 1846), *Cyclopecten pernomus* (Hertlein, 1935), *Nuculana lobula* (Dall, 1908), *Parvilucina mazatlanica* (Carpenter, 1857), and *Gouldia californica* Dall, 1917. The bathymetric patterns in the abundance and species composition of the bivalve community and their relationship to environmental parameters are discussed. The structure of the assemblages differed with depth, with peak abundances and species richness (1) between 24 and 40 m with medium sand and sandy silt substrata and (2) at intermediate depths between 71 and 74 m, with sandy silt and silty clay substrata. The species characterizing shallow, intermediate, and deep zones were the most abundant or those exclusive of each zone. Diversity, dominance, and evenness decreased at the deeper stations. The distinctive species composition of these zones may be the result of variation in depth, oxygen concentration, and substratum.

Key words: Bathymetric patterns, diversity, dominance, evenness, benthos

Our understanding of the taxonomic composition of the bivalve fauna of the Mexican Pacific stems from the exhaustive survey of Keen (1971) and the coverage of some Panamic taxa by Keen and Coan (1974) and Coan *et al.* (2000). The literature reviews of Skoglund (1991, 2001) are also important since they cite new species, redefine taxonomic relationships, and provide new records and range extensions for many bivalves from the Panamic Province.

Most of the ecological literature on benthic communities from the Mexican Pacific refers to the Gulf of California. Parker (1964) reviewed the early biological exploration in the Gulf of California and provided an extensive description of macroinvertebrate assemblages and their environments. Parker provided a list of more than 380 species of bivalves and assemblages from intertidal and shallow rocky or sandy shores to the abyssal basin and outer continental slope, to 4,122 m depth. Coan (1968) described the shallow benthic mollusc community (0-49 m) at Bahía de Los Angeles, located on the northeastern coast of Baja California, and concluded there was a single assemblage, typical of a silty-sand substratum in semi-protected bays of tropical and subtropical areas. Zamorano and Hendrickx (2007) reported a total of 56 species of deep-water molluscs collected during the TALUD IV-IX cruises in depths >500 m in the southern Gulf of California. The most recent biodiversity model of marine and brackish-water Mollusca from the Gulf of California, proposed by Hendrickx *et al.* (2007), analyzed the latitudinal and bathymetric distribution of 2,194 species, including 565 bivalves. Additionally, some catalogues of records of biological collections include bivalves from the Gulf of California (Morris 1966, Abbott 1974, Hendrickx and Toledano-Granados 1994, Hendrickx and Brusca 2002).

Surveys of mollusc communities from the Mexican Central Pacific are scarce, non-continuous, and have been carried out mostly in the intertidal and shallow subtidal zones. In the coast of Jalisco and Colima, only a few works focus mainly on the taxonomic composition and abundance of gastropods (Ríos-Jara *et al.* 1996, Pérez-Peña and Ríos-Jara 1998), scaphopods (Ríos-Jara *et al.* 2003a, 2003b), or both gastropod and bivalve communities (Landa-Jaime and Arciniega-Flores 1998, Ríos-Jara *et al.* 2001) with very few records of bivalves. Holguín-Quiñones and González-Pedraza (1994) provide the only catalogue of molluscs for this region and include 87 species of bivalves mostly from rocky and sandy beaches and shallow subtidal areas to 39 m depth.

The Atlas expeditions off the coast of Jalisco and Colima in 1988 resulted in one of the most important and most extensive collections to date of benthic marine molluscs from the Mexican Central Pacific. The R/V *El Puma* of the

Universidad Nacional Autónoma de México extensively sampled the shelf of both states, from Puerto Vallarta to the southern limit of Colima. The specimens were deposited in the collection of the Laboratorio de Ecosistemas Marinos y Acuicultura of the Universidad de Guadalajara in the city of Guadalajara and have resulted in reports mostly related to the gastropods and the scaphopods (Ríos-Jara *et al.* 1996, 2001, 2003a, 2003b, Pérez-Peña and Ríos-Jara 1998). The present study examines the bivalves collected during the Atlas V expedition along the continental shelf of Jalisco and Colima. Taxonomic changes are updated, and range extensions reviewed with an analysis of distribution patterns with respect to depth, oxygen concentration, and substratum.

MATERIALS AND METHODS

Study area

The area of study is the narrow (7-10 km) continental shelf off the states of Jalisco and Colima, along the Pacific coast of Mexico (Fig. 1) with an area of approx. 5, 315 km^2 (Ruíz-Durá 1985). The area extends *ca.* 364 km of coastline, from the mouth of the Río Ameca, Bahía Banderas (20°39′N), to the mouth of the Río Cohuayana (18°39′N).

This region has irregular topography, with foothills and mountains which form cliffs, bays, estuaries, and beaches of diverse sizes and shapes. *Ca.* 70% of the coastline is sandy beaches; rocky areas are mixed with sands and include volcanic rock platforms, boulders, stones, and pebbles. On the sea bottom, areas of uneven topography intercalate with relatively flat zones (Galavíz-Solis and Gutiérrez-Estrada 1978, Guzmán-Arroyo and Flores-Rosas 1988). The composition of sediments in the continental shelf of the tropical Mexican Pacific is mostly terrigenous and pelagic clays with small areas of calcareous and bio-siliceous mud and silts (McCoy and Sancetta 1985). This coastal region includes several rivers, coastal lagoons, and estuaries.

Sampling methods

On board the R/V *El Puma*, topography and depth were determined continuously with an Edowestern analog echo-

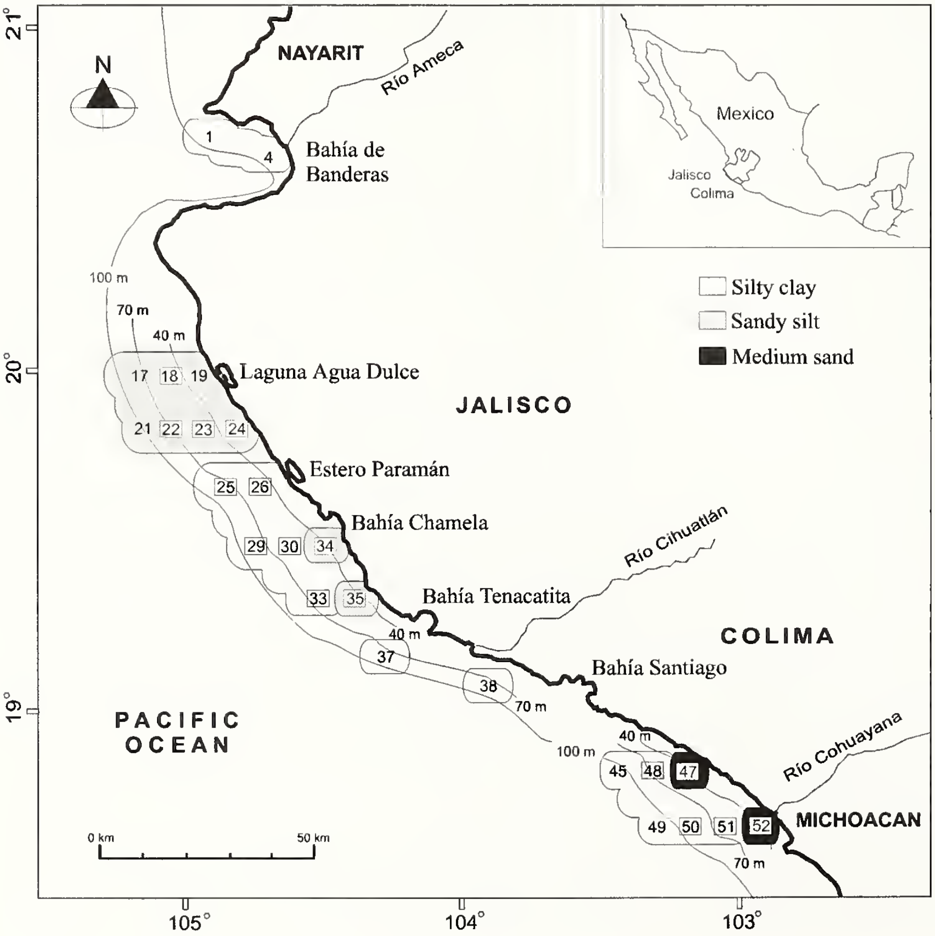
21°
20°
19°
N
NAYARIT
Río Ameca
1
4
Bahía de Banderas
Mexico
Jalisco
Colima
100 m
70 m
40 m
17
18
19
Laguna Agua Dulce
JALISCO
21
22
23
24
Estero Paramán
25
26
Bahía Chamela
29
30
34
33
35
Bahía Tenacatita
Río Cihuatlán
40 m
37
COLIMA
Bahía Santiago
38
70 m
PACIFIC OCEAN
100 m
40 m
45
48
47
Río Cohuayana
49
50
51
52
MICHOACAN
70 m
0 km
50 km
105°
104°
103°
☐ Silty clay
☐ Sandy silt
■ Medium sand

Figure 1. Location of sampling stations and types of substratum along the coast of Jalisco and Colima, Mexican Central Pacific. The stations with a square indicate those where bivalves were collected. The isolines at 40, 70, and 100 m indicate the shallow (18-40 m), intermediate (48-74 m), and deep (83-129 m) bathymetric zones in which sampling stations were grouped.

sounder system. Thirteen transects perpendicular to the coastline were established at intervals of ~10′ of latitude (Fig. 1). One to four sampling stations were conducted on each transect, for a total of 25 samples collected with a Van Veen grab. The grab collected 20 L of sediment in a surface area of 0.1 m^2. At each station, two grab samples were taken. The depth ranged from 18 to 129 m. Sediments were sieved through three screens (mesh size = 10, 3, and 1 mm) and analyzed in the Instituto de Geografía of the Universidad de Guadalajara, México.

Living bivalves were preserved in 70% ethanol. Individuals were identified to species using Morris (1966), Keen (1971), Abbott (1974), and Coan *et al.* (2000). Only shells that permitted clear identification were used. Taxonomy and distributional ranges were updated using Skoglund's publications (1991, 2001).

There are at least three types of substrata in the area (Pérez-Peña and Ríos-Jara 1998): (1) silty clay (most particles <0.02 mm), (2) sandy silt (0.02-0.015 mm), and (3) medium sand (>0.2 mm). Greater heterogeneity of the sediments occurred in shallow sampling stations (18-60 m) compared to the intermediate (48-74 m) and deeper stations. Sediments were more homogeneous at deeper stations than in the intermediate and shallow stations, and decreased in particle size from medium sand and sandy silt to silty clay.

To analyze the effect of depth and substratum on the distribution and abundance of the bivalves, sampling stations were grouped in (1) a shallow zone (SZ) (18-40 m) with coarse substratum (medium sand and sandy silt), which includes stations 52, 24, and 47; (2) a transition zone with intermediate depths (IZ) (48-74 m), sandy silt and silty clay, including stations 35, 23, 48, 26, 51, 34, 18, 22, and 30; and (3) a deep zone (DZ) (83-129 m) with homogeneous substratum made of silty clay, including stations 33, 50, 38, 25, 29, and 37.

Preliminary analysis revealed that abundance values were asymmetrical (X^2 goodness-of-fit statistic, $P < 0.01$), and sample variances were not homogeneous (Hartley's F_{max} test; Byrkit 1987) (Statgraphics plus 5.0 computer program). Therefore, the non-parametric Kruskal-Wallis test (Sokal and Rohlf 1989) was used to analyze bathymetric patterns of bivalve abundance. Significant differences were further analyzed using the Bonferroni test to identify differences of bivalve abundance means among sampling stations.

The structure of the bivalve community was analyzed by estimating ecological indices for the three bathymetric zones (SZ, IZ, and DZ) using the computer program Species Diversity and Richness III (1998). Diversity was estimated by means of the Shannon-Weaver index (H′) (Magurran 1988), species richness with the Margaleff Index (D_{Mg}) (Magurran 1988), dominance with the Simpson Index (D′) (Simpson 1949), and evenness with the Pielou Index (J′) (Pielou 1977). Differences in these indices among zones were determined with a Kruskal-Wallis test; significant differences were further analyzed using the *a posteriori* test of Bonferroni.

Pearson correlation coefficients (Minitab computer program; Sokal and Rohlf 1989) were used to determine the relationship between environmental (temperature, depth, and oxygen concentration) and biological parameters (abundance and number of species). The Pearson probability *P*-values were used to determine any significant correlations.

RESULTS

Composition and abundance

Bivalves were found at 16 of the 25 sampling stations, at depths between 18 and 112 m (Fig. 1). Most of these stations had silty clay (8 stations) and sandy silt (6 stations) substrata; only two stations had medium sand. A total of 5,196 individuals belonging to 28 families, 59 genera, and 95 species was collected (Appendix 1). The number of species per family varies considerably (from 1 to 15). Twelve families (42.85%) contain a single species, while the most diverse families contain nine or more species: Veneridae (15), Tellinidae (13), Lucinidae (10), and Arcidae (9).

The density of bivalves obtained with the grab ranged between 13,015 individuals /m^2 in station 47 and 15 individuals/m^2 in station 48 (mean density per station = 1,623.75). The number of species varied between 1 and 63 species/station (mean = 6) (Appendix 1). There was considerable variation in the structure of the bivalve communities along the continental shelf, with areas of high and low density of bivalves, and notable differences in the number and composition of species. In general, the zones with higher numbers of individuals coincide with those of high species richness (Fig. 2).

The density of individuals and species decrease at depths between 48 and 66 m of the Intermediate Zone (IZ) with 15-210 individuals/m^2 and 3-16 species/station, and in the Deep Zone (DZ) (83-112 m), with 55-2070 individuals/m^2 and only 1-11 species/station. Notably, the boundaries of these zones present areas with peaks of very high values: (1) in station 47 (40 m) between SZ and IZ, with total of 13,015 individuals/m^2 and 63 species and (2) in station 30 (74 m) between the IZ and DZ, with 5,585 individuals/m^2 and 28 species. These two areas include the majority of all bivalves collected (50.1% and 34.5% of all individuals, respectively) and a large fraction of the species (65.6% and 47.9%).

The assemblages of bivalve species across the continental shelf of Jalisco and Colima thus show a tendency to decrease in the abundance and number of species from the shallow to the deeper areas. However, there is not statistical

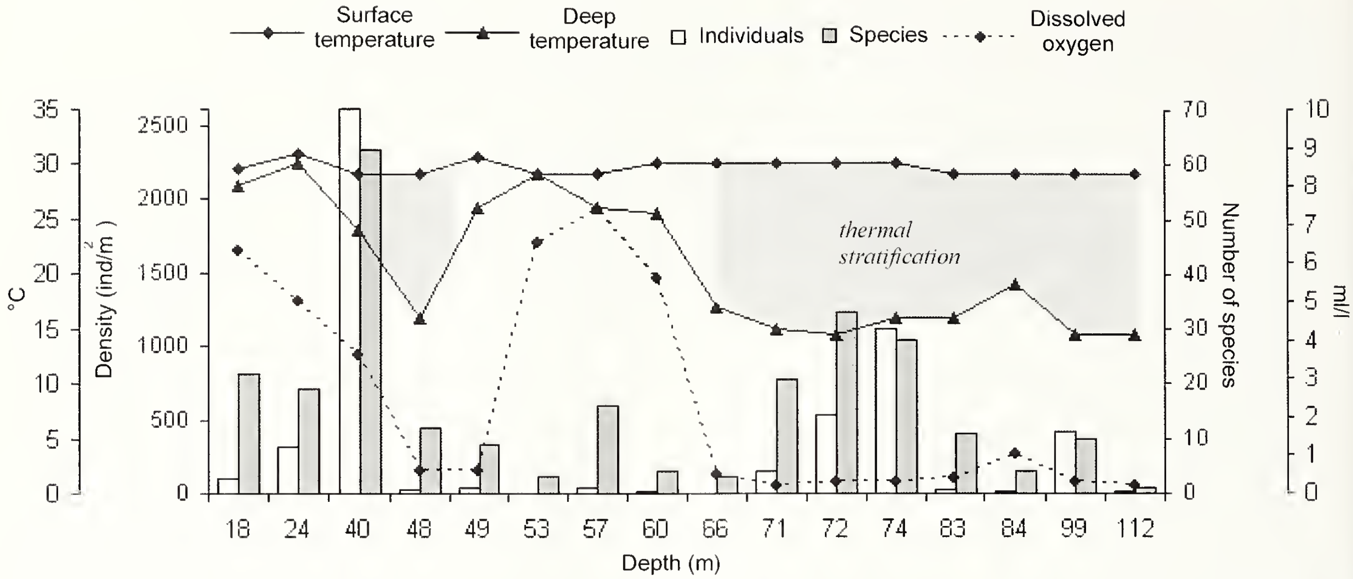

Figure 2. Density (individuals per m^2) and number of species collected with a Van Veen grab in the continental platform of Jalisco and Colima, México. The differences between surface and bottom temperatures during the sampling period define zones along the continental shelf with thermal stratification. Variation in dissolved oxygen concentration is also indicated.

difference in the abundance of bivalves between the bathymetric zones (Kruskal-Wallis test, $N = 3$, $P = 0.23$) (Table 3).

Relationship between environmental and biological parameters

Highly significant negative correlations occurred between depth and temperature (Pearson correlation, $r = -0.76$, $N = 16$, $P = 0.0003$) and depth and oxygen concentration ($r = -0.638$, $N = 16$, $P = 0.009$). Minimal values of temperature and oxygen occurred in areas deeper than 60 m although several peaks of both parameters are evident at depths of 18-24 m, 53-60 m, 84 m. Consequently, bottom water temperature was significantly correlated with oxygen concentration ($r = 0.874$, $N = 16$, $P = 0.00009$). Depth was significantly correlated with the number of species of bivalves ($r = -0.471$, $N = 16$, $P = 0.049$). Low oxygen concentrations overlap the areas of strong thermal stratification at 48 m and between 66 and 112 m depth. Oxygen levels increase considerably between 53 and 60 m where there is no apparent stratification. Continuous hypoxic conditions occur in areas deeper than 60 m but become more severe at station 29 where the oxygen level falls to 0.2 ml/l and only one species was collected (Fig. 2).

Because the peaks in the number of individuals and species closely coincide across the continental shelf (Fig. 2), both variables had a significant positive correlation coefficient ($r = 0.740$, $N = 16$, $P = 0.0005$). However, no significant correlation was found between depth and abundance ($r = -0.123$, $N = 16$, $P = 0.627$) probably because of the large increment in the number of bivalves registered in the station 25 at 99 m, especially the clams *Corbula ira* (242) and *Nuculana lobula* (149). The correlation coefficients for all other pairwise comparisons were low and not statistically significant ($P > 0.05$).

Twelve species were very common (>100 individuals) in the samples, representing 78.16% of all individuals (Table 1). These dominant species had a widespread bathymetric distribution across the continental shelf, but most individuals concentrated in high numbers in one or two stations either in the shallow (SZ) or deeper stations (DZ). This is more evident in the SZ, where nine dominant species represented 71% of the bivalve abundance and in the DZ where two species represent 91% of the abundance (Table 2). The observed decrease in the total bivalve density in the IZ is largely the result of the decline in the populations of the 12 most abundant species.

The shallow zone (18-40 m) contains an assemblage of 79 species. Eleven species are considered dominant (Table 2). These species are semi-infaunal or infaunal, which can be attributed to the sediment type, mostly medium sand (67%) and sandy silt (33%). The only epifaunal species in this group, *Cyclopecten pernomus*, was found in great numbers in medium sand substratum at 40 m. The family with the most species was Veneridae (3); each of the other eight dominant species belongs to different families. The stations with this assemblage of species are in Colima (52 and 47) and in the

Table 1. Abundance, habitat, and feeding habits of the most representative bivalve species. MS, medium sand; SS, sandy silt; SC, silty clay; DF, deposit feeder; FF, filter feeder.

Species	Abundance (total individuals)	Relative abundance (%)	Cumulative relative abundance (%)	Type of substratum	Depth range (m)	Habitat	Feeding habit
1. *Nuculana laeviradius*	690	13.28	13.28	MS, SS, SC	18-84	Infaunal	DF
2. *Crassinella pacifica*	680	13.09	26.37	MS, SS, SC	18-74	Infaunal	—
3. *Corbula nasuta*	535	10.30	36.66	MS, SS, SC	18-74	Infaunal	FF
4. *Anadara adamsi*	435	8.37	45.03	MS, SS, SC	24-94	Semi-infaunal	FF
5. *Parvilucina approximata*	399	7.68	52.71	MS, SS, SC	18-83	Infaunal	FF
6. *Nucula declivis*	263	5.06	57.78	MS, SS, SC	18-83	Infaunal	DF
7. *Corbula ira*	242	4.66	62.43	SC	99	Infaunal	—
8. *Radiolucina cancellaris*	208	4.00	66.44	MS, SS, SC	40-83	Infaunal	—
9. *Cyclopecten pernomus*	188	3.62	70.05	MS, SS, SC	24-74	Epifaunal	FF
10. *Nuculana lobula*	173	3.33	73.38	MS, SS, SC	18-112	Infaunal	DF
11. *Parvilucina mazatlanica*	144	2.77	76.15	MS, SS, SC	18-83	Infaunal	DF
12. *Gouldia californica*	104	2.00	78.16	MS, SS	40-72	Infaunal	FF

middle portion of Jalisco (24), but the assemblage possibly extends to all shallow areas with similar conditions along the length of the tropical Mexican Pacific. Many species (32) are found only in this zone, which indicates this is an optimum habitat for the bivalve species. The black clam *Megapitaria squalida* (G. B. Sowerby I, 1835) was the only dominant exclusive to this zone and together with other abundant species [*Corbula nasuta, Anadara adamsi, Cyclopecten pernomus, Gouldia californica, Chione compta* (Broderip, 1835), and *Semelina campbellorum* Coan, 2003] characterizes this assemblage (Table 2).

The intermediate zone assemblage (48-74 m) is distinct and is characterized by several species which are dominant only in this zone: *Parvilucina approximata, Parvilucina mazatlanica, Lucinisca centrifuga* (Dall, 1901), and *Nuculana acapulcensis* (Pilsbry and Lowe, 1932) (Table 2). The family with the most dominant species (4) was Lucinidae followed by Nuculanidae (2). The zone has finer sediments than the SZ (mostly sandy silt and silty clay), and most of the dominant species are also semi-infaunal or infaunal. Although five dominant species were also dominant in the shallow zone, there is also a considerable number of exclusive species (10). Some of these species were also found in deeper waters, until 83 m. The total number of species (55) and individuals (1,896) decrease with respect to shallower areas although both species and individuals are considerably higher between 71 and 74 m.

All samples from stations in the outer shelf, 83 to 112 meters, were taken on silty clay bottom. This deeper zone contained a rather different assemblage of bivalve species from those found in the shallow and intermediate zones. This was the zone with the lowest number of bivalve species (22) although three were exclusive to this zone. Although only two species, *Nuculana lobula* and *Corbula ira*, may be considered dominant because of their high relative abundance, it is notable that *C. ira* is also exclusive to this zone.

Species richness, diversity, dominance, and evenness

The shallow and intermediate zones have similar values for the ecological indices, but they all decrease toward the deep zone. However, significant differences were found only in species richness and diversity among the bathymetric zones (Kruskal-Wallis test, $N = 3$, $P < 0.05$) (Table 3). The Bonferroni multiple-comparison test revealed that the values for the SZ and IZ were not statistically different but both were greater than in the DZ.

DISCUSSION

Although bivalves play a key role in the macroinvertebrate community of the intertidal zone, there have been few attempts to relate benthic bivalves to environmental factors in the continental shelf of Jalisco and Colima. Trawling nets used in other studies do not collect the smaller semi-infaunal and infaunal species which comprise this group (Landa-Jaime and Arciniega-Flores 1998, Godínez-Domínguez and Gonzalez-Sanson 1999). These studies thus underestimate their importance in the benthos. During the Atlas V expedition, the fauna from grab samples included many gastropods (Perez-Peña 1989) and some scaphopods (Ríos-Jara *et al.* 2003a) but bivalves represented the greatest numbers of individuals and species.

Because most species were found in more than one bathymetric zone, specific assemblages were characterized by the dominant species and by those found in only one zone.

Considerable changes in species composition and dominance occur because the most abundant species were frequently collected in high numbers (>100 individuals) but only at one or two sampling stations. Species with a wide bathymetric distribution tend to be found either in the shallow or the deep zone, forming aggregations at specific sampling stations. In addition, even though bivalve abundance can be highly variable, some predictions of community structure can be made based on a few key environmental factors such as type of substratum and oxygen concentration.

Thorson (1957) proposed that parallel bottom communities, characterized by closely related genera, exist where environmental conditions are similar. Parker (1964) found similar species assemblages, including bivalves, in areas with similar conditions, as predicted by Thorson (1957). Other examples of parallel communities have also been described (*e.g.*, Coull and Herman 1970, Asakura and Suzuki 1987, Chertoprud *et al.* 2007). However, there are also examples that do not support the parallel communities hypothesis (Gallardo 2003). A comparison of the bivalve assemblage described by Parker (1964) from near-shore (11-26 m) with sand to sand-mud substrata of the Gulf of California to the bivalve assemblage of the shallow-water zone (18-40 m) with medium sand and sandy silt of Jalisco and Colima indicates some similarities. In both regions, the majority of species live in semi-infaunal and infaunal habitats, and the most diverse families are Nuculanidae and Veneridae. In addition, *Megapitaria squalida* is very abundant, several species of the genera *Nuculana* Link, 1807, *Anadara* J. E. Gray, 1847 and *Chione* Megerle von Mühlfeld, 1811 are dominant in both assemblages, and both share the same three most diverse families (Veneridae, Tellinidae, and Arcidae) (Hendrickx *et al.* 2007). Other comparisons among

Table 2. Most abundant and exclusive species in the shallow, intermediate, and deep zones. The most abundant species of each list contain ≥80% of all individuals within each zone.

Shallow zone 18-40 m 67% medium sand, 33% sandy silt	Intermediate zone 41-80 m 56% sandy silt, 44% silty clay	Deep zone 81-129 m 100% silty clay
Most abundant species		
1. *Corbula nasuta*	1. *Parvilucina aproxímata*	1. *Corbula ira*
2. *Nuculana laeviradius*	2. *Crassinella pacifica*	2. *Nuculana lobula*
3. *Anadara adamsi*	3. *Nuculana laeviradius*	
4. *Crassinella pacifica*	4. *Radiolucina cancellaris*	
5. *Nucula declivis*	5. *Parvilucina mazatlanica*	
6. *Cyclopecten pernomus*	6. *Lucinisca centrifuga*	
7. *Megapitaria squalida*	7. *Nuculana acapulcensis*	
8. *Gouldia californica*	8. *Nucula declivis*	
9. *Chione compta*	9. *Cyclopecten pernomus*	
10. *Radiolucina cancellaris*		
11. *Semelina campbellorum*		
Exclusive species		
1. *Anadara aequatorialis*	1. *Anadara obesa*	1. *Corbula ira*
2. *Anadara nux*	2. *Barbatia reeveana*	2. *Kellia suborbicularis*
3. *Chione pulicaria*	3. *Conchocele excavata*	3. *Strophocardia megastropha*
4. *Corbula marmorata*	4. *Crassinella varians*	
5. *Crassinella ecuadoriana*	5. *Lucinisca fenestrata*	
6. *Donax gracilis*	6. *Macoma siliqua*	
7. *Dosinia dunkeri*	7. *Nucula schenki*	
8. *Isognomon recognitus*	8. *Pitar aletes*	
9. *Laevicardium elenense*	9. *Pitar berryi*	
10. *Lirophora mariae*	10. *Tellina pristiphora*	
11. *Lucina prolongata*		
12. *Lunarca brevifrons*		
13. *Mactrellona subalata*		
14. *Megapitaria squalida*		
15. *Lirophora kellettii*		
16. *Pitar concinnus*		
17. *Plicatula pencillata*		
18. *Psammotreta aurora*		
19. *Semele pallida*		
20. *Semele verrucosa*		
21. *Sheldonella olssoni*		
22. *Strigilla cicercula*		
23. *Strigilla dichotoma*		
24. *Strigilla sp.*		
25. *Tagelus politus*		
26. *Tellina pacifica*		
27. *Trachycardium belcheri*		
28. *Trachycardium procerum*		
29. *Trachycardium senticosum*		
30. *Transennella modesta*		
31. *Trigoniocardia granifera*		

Table 3. Ecological indices estimated for the bivalve communities from three bathymetric zones of the continental shelf of Jalisco and Colima, México. H′ = Shannon-Weaver's diversity index, D_{Mg} = Margaleff's species richness index, D′ = Simpson's dominance index and J′ = Pielou's evenness index. Significant differences among zones (*) were found for the Species richness (D_{Mg}) and Diversity (H′) indices (Kruskal-Wallis Test, $P < 0.05$) and were further analyzed using the Bonferroni test.

	Shallow zone (SZ)	Intermediate zone (IZ)	Deep zone (DZ)	Kruskal-Wallis test (H value)	Bonferroni test among zones
Total number of individuals (N)	2834	1896	466	2.41	
Total number of species (S)	79	55	22		
Species richness (D_{Mg})	9.81	7.15	3.41	6.81*	SZ = IZ > DZ
Diversity (H′)	2.86	2.63	1.32	6.58*	SZ = IZ > DZ
Dominance (D′)	10.28	8.91	2.54	4.16	
Evenness (J′)	0.62	0.57	0.28	1.61	

the bivalve assemblages of Jalisco and Colima and those described by Parker (1964) show fewer similarities. The physical characteristics of these environments and the biology of the species provide explanations for the presence of distinct bivalve assemblages in the different zones across the continental shelf of Jalisco and Colima. A reduction in macrofaunal diversity with depth was also observed in the molluscs from the Gulf of California (Hendrickx *et al.* 2007). Other studies, however, showed no significant relationship between diversity and depth for the demersal invertebrate communities of the southern continental shelf of Jalisco (Landa-Jaime and Arciniega-Flores 1998, Godínez-Dominguez and Gonzalez-Sanson 1999).

The abundance of bivalves depends on their affinity to type of substratum, temperature, depth, and oxygen concentration (Levin *et al.* 2001). The structure and composition of soft-sediment communities are related to sediment characteristics (*e.g.*, Sanders 1968, Gray 1981, Snelgrove and Butman 1994); the type of substratum is particularly important since all these species live on or within the sediments. However, the presence of some species or even the whole assemblages may also be determined indirectly by biological factors, such as feeding mechanisms, competition for food, predator-prey relationships, etc. In the present study, the abundance and distribution of bivalves was related to the type of substratum and feeding habit. Some of the most abundant species, such as the infaunal, filter feeding *Corbula ira*, *Nuculana lobula*, and *Parvilucina approximata* were more common in finer substrata, while some semi-infaunal (*Anadara adamsi*) and epifaunal (*Cyclopecten pernomus*) filter feeding species were characteristic of coarser substrata. In general, filter feeding was important for most of the bivalve species, and was more common in shallow epibenthic and semi-infaunal habitats.

In the continental shelf of the tropical Mexican Pacific, the irregular topography of the coastline is closely related to the different substrata, predominantly of terrigenous origin, found in this region (McCoy and Sancetta 1985). The spatial variation in species diversity is correlated with the heterogeneity of sediment grain size across the continental shelf. The greater heterogeneity in particle size and texture of the shallow areas probably offers a greater variety of benthic habitats. Species composition across the continental shelf was closely related to the vertical distribution of the sediments. Most bivalves were collected in the sandy silt and silty clay substrata of the shallow zone (18-40 m). The majority of these species are filter feeders or deposit feeders that rely on the infaunal or semi-infaunal habitats of the sea floor. Some other representative species of this zone are epifaunal filter feeders including those of the families Arcidae, Chamidae, Mytilidae, Pectinidae, and Plicatulidae. Among the most important are the ark shell *Arca pacifica* (G. B. Sowerby I, 1833), the so called "pata de mula" *Anadara* spp., the Pacific chama *Chama sordida* Broderip, 1835, the scallop *Argopecten ventricosus* (G. B. Sowerby II, 1842) and the mother-of-pearl *Pteria sterna* (Gould, 1851). These species live attached to hard substrata, other shells, rocks, or even fixed by the left valve (*C. sordida*).

In the intermediate and deep zones, sediments are finer and more homogeneous than in the shallow zone and the number of species decreases. The number of epifaunal species also decreases, and most are infaunal deposit or filter feeders, with a single semi-infaunal species of carrion feeder (Verticoriidae). In the deep zone, the number of species is even lower and dominance increases because two infaunal species, *Corbula ira* and *Nuculana lobula*, represent 31% of all individuals in this zone.

Bottom water temperatures apparently had little effect on the abundance and distribution of bivalves; the sampling stations with the greatest abundance had temperatures within a wide range of values. However, the differences between surface and bottom water temperatures are probably important because thermal stratification may control oxygen and food supply from surface waters. Benthic biomass and abundance are assumed to reflect the rate of nutrient input to the seafloor. Other variables related to thermal stratification are the hydrodynamic regime and the water circulation. Bottom water oxygen concentrations in the continental shelf of the Mexican Tropical Pacific vary from high values (~5 ml/l) in the shallow areas <100 m when the circulation is strong enough to maintain high concentrations or little oxygen consumption in the bottom layer, to low values (0.25 ml/l) toward deeper areas where there is weak circulation (Pacheco-Sandoval 1991). In the area of study, prolonged periods of hypoxic conditions may eliminate most macrobenthos from the seabed (they either emigrate from the area or die). A hypoxia-stressed benthos is typified by short-lived, smaller surface deposit-feeding polychaetes and the absence of bivalves (Rabalais *et al.* 2002). Thus, peaks in bivalve abundance occur in the areas of strong stratification where the availability of dissolved oxygen probably is not a limiting factor.

In the eastern Pacific Ocean there are extensive midwater regions where oxygen is depleted, typically between 100 and 1,200 m depth (Oxygen Minimum Zone, OMZ) (Kamykowsky and Zentara 1990, Levin *et al.* 2001). In shallow areas, OMZ may be evident at depths lower than 100 m. Where these low oxygen regions intercept the continental seabed, the benthos experiences hypoxia (Gallardo 1985, Levin *et al.* 1991) and reduced macrofaunal diversity (Parker 1964, Mullins *et al.* 1985, Levin *et al.* 1991). Our data for the continental shelf of Jalisco and Colima coincide with this general pattern.

Although oxygen exerts a strong effect on species richness, organic matter has a greater influence on dominance (Levin and Cage 1998). Together these factors lower diversity within the OMZs. Significant reduction of macrofaunal species richness by low oxygen may not occur until concentrations fall below 0.4 or 0.3 ml/l; this value may be ever lower for those species tolerant to hypoxia (Levin *et al.* 2001). Among the macrofauna, many molluscs appear less tolerant of hypoxia than other taxa (Diaz and Rosenberg 1995) although there are some exceptions. In the continental shelf of Jalisco and Colima, the number of species decreases in the deep zone where oxygen concentrations fall to ≤0.3 ml/l. However, such species as *Corbula ira* and *Nuculana lobula* are very abundant and cause an overall decrease in diversity. Members of the family Lucinidae seem particularly widespread at this zone. The affinity of lucinid bivalves to numerous OMZ sites within the eastern Pacific has been documented by Levin *et al.* (2001).

In summary, the results suggest that substratum variability, oxygen concentration, and thermal stratification have an important influence on the bathymetric distribution and abundance of bivalves in the continental shelf of Jalisco and Colima, and that the bivalve assemblages are not simply the result of species independently sorting in diverse environments. This study also shows that, even under hypoxic conditions, bivalves may display significant peaks of abundance. During the Atlas V expedition the abundance and the number of species of gastropods and scaphopods also decreased with depth and no live specimens were collected at stations deeper than 83 m (Perez-Peña and Ríos-Jara 1998, Ríos-Jara *et al.* 2003b). Low dissolved oxygen concentrations have also been mentioned as limiting the distribution of benthic molluscs from the Gulf of California (Guerrero-Pelcastre 1986) and the continental platform of Guerrero (Lesser-Hiriart 1984). In the present study, the dominant species were used to characterize the bathymetric zones; however, the importance of many less common or even rare species should also be taken into consideration because they determine the structure of the community.

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Appendix 1. Density (individuals/m^2) of bivalve species obtained with a Van Veen grab in the continental platform of Jalisco and Colima, México. MS, medium sand; SS, sandy silt; SC, silty clay.

Sampling station	52	24	47	35	23	48	26	51	34	18	22	30	33	50	25	29	Mean number of individuals per m^2
Type of substratum	MS	SS	MS	SS	SS	SC	SC	SC	SS	SS	SS	SC	SC	SC	SC	SC	
Depth (m)	18	24	40	48	49	53	57	60	66	71	72	74	83	84	99	112	
Temperature (°C)	28	30	24	16	26	29	26	25.5	17	15	14.5	16	16	19	14.5	14.5	
Dissolved oxygen (ml/l)	6.3	5.0	3.6	0.6	0.6	6.5	7.4	5.6	0.5	0.2	0.3	0.3	0.4	1.0	0.3	0.2	
Family Nuculidae																	
1. *Nucula declivis* Hinds, 1843	25		895	45	5		5			5	215	115	5				82.18
2. *Nucula schenki* Hertlein and Strong, 1940				5	5						80	10					6.25
Family Nuculanidae																	
3. *Nuculana acapulcensis* (Pilsbry and Lowe, 1932)	5			5	65		20		5	85	125	110	5				26.56
4. *Nuculana laeviradius* (Pilsbry and Lowe, 1932)	50	15	2235	5		5		10		25	155	940	5	5			215.62
5. *Nuculana lobula* (Dall, 1908)	5									35	10		15		745	55	50.06
Family Arcidae																	
6. *Acar gradata* (Broderip and G. B. Sowerby I, 1829)		5			5												0.625
7. *Anadara adamsi* Olsson, 1961		5	1900		90						85	95					135.93
8. *Anadara aequatorialis* (d'Orbigny, 1846)			30														1.87
9. *Anadara concinna* (G. B. Sowerby I, 1833)			110							5	5						7.5
10. *Anadara formosa* (G. B. Sowerby I, 1833)			5														0.31
11. *Anadara obesa* (G. B. Sowerby I, 1833)												40					2.5
12. *Arca pacifica* (G. B. Sowerby I, 1833)		5													10		0.94
13. *Barbatia reeveana* (d'Orbigny, 1846)							5										0.31
14. *Lunarca brevifrons* (G. B. Sowerby I, 1833)			5														0.31
Family Noetiidae																	
15. *Sheldonella delgada* (Lowe, 1935)			35		5						10	50	5		5		6.56
16. *Sheldonella olssoni* (Sheldon and Marry, 1922)		30															1.87
Family Glycymerididae																	
17. *Tucetona multicostata* (G. B. Sowerby I, 1833)		5													15		1.25
18. *Tucetona strigilata* (G. B. Sowerby I, 1833)		5	15								5						1.56
Family Mytilidae																	
19. *Crenella decussata* (Montagu, 1808)			225								10	45					18.44
Family Pteriidae																	
20. *Pteria sterna* (Gould, 1851)			10														0.625
Family Isognomonidae																	
21. *Isognomon recognitus* (Mabille, 1895)		90															5.625
Family Pectinidae																	
22. *Argopecten ventricosus* (G. B. Sowerby II, 1842)			105		5		5				5				5		7.812
23. *Leptopecten biolleyi* (Hertlein and Strong, 1946)			20								10	20					3.125
24. *Leptopecten velero* (Hertlein, 1935)		5	20									5					1.875
Family Propeammusiidae																	
25. *Cyclopecten pernomus* (Hertlein, 1935)		5	565		20						90	260					58.75
Family Plicatulidae																	
26. *Plicatula pencillata* Carpenter, 1857	5	35	25														4.06
Family Crassatellidae																	
27. *Crassinella adamsi* Olsson, 1961			30				5				5						2.5
28. *Crassinella ecuadoriana* Olsson, 1961		10															0.625
29. *Crassinella pacifica* (C. B. Adams, 1852)	45		1505	5			10				625	1180	15				212.5
30. *Crassinella varians* (Carpenter, 1857)												15					0.94

Appendix 1. (continued)

Sampling station	52	24	47	35	23	48	26	51	34	18	22	30	33	50	25	29	
Type of substratum	MS	SS	MS	SS	SS	SC	SC	SC	SS	SS	SS	SC	SC	SC	SC	SC	Mean number of individuals per m^2
Depth (m)	18	24	40	48	49	53	57	60	66	71	72	74	83	84	99	112	
Temperature (°C)	28	30	24	16	26	29	26	25.5	17	15	14.5	16	16	19	14.5	14.5	
Dissolved oxygen (ml/l)	6.3	5.0	3.6	0.6	0.6	6.5	7.4	5.6	0.5	0.2	0.3	0.3	0.4	1.0	0.3	0.2	
Family Carditidae																	
31. *Cardites laticostata* (G. B. Sowerby I, 1833)		55															3.44
32. *Cyclocardia beebei* (Hertlein, 1958)											5				50		3.44
33. *Strophocardia megastropha* (Gray, 1825)															10		0.625
Family Lucinidae																	
34. *Divalinga perparvula* (Dall, 1901)			30							10							2.5
35. *Lucina prolongata* (Carpenter, 1857)			10														0.625
36. *Lucinisca centrifuga* (Dall, 1901)			10	5			20			190	70	155	5				28.44
37. *Lucinisca fenestrata* (Hinds, 1845)												10					0.625
38. *Lucinoma annulatum* (Reeve, 1850)			5				5			10		40	10				4.375
39. *Neophysema aphanes* Taylor and Glover, 2005											45			50			5.93
40. *Parvilucina approximata* (Dall, 1901)		5	75	10						85		1815	5				124.69
41. *Parvilucina mazatlanica* (Carpenter, 1857)	35		5						10	125	520			25			45
42. *Pegophysema edentuloides* (Verrill, 1870)									5		5				5		0.94
43. *Radiolucina cancellaris* (Philippi, 1846)			295	5				15		70	350	295	10				65
Family Ungulinidae																	
44. *Diplodonta soror* (C. B. Adams, 1852)			15				5			10	5						2.19
45. *Diplodonta subquadrata* (Carpenter, 1856)	5										10						0.94
Family Thyasiridae																	
46. *Thyasira flexuosa* (Montagu, 1803)												40					2.5
Family Kellidae																	
47. *Kellia suborbicularis* (Montagu, 1803)														5			0.31
Family Chamidae																	
48. *Chama sordida* Broderip, 1835															15		094
Family Cardiidae																	
49. *Laevicardium elenense* (G. B. Sowerby II, 1841)			5														0.31
50. *Trachycardium belcheri* (Broderip and G. B. Sowerby I, 1829)	5		5														0.625
51. *Trachycardium procerum* (G B. Sowerby I, 1833)	5		5														0.625
52. *Trachycardium senticosum* (G. B. Sowerby I, 1833)			55														3.44
53. *Trigoniocardia granifera* (Broderip and G. B. Sowerby I, 1829)	5		85														5.63
54. *Trigoniocardia obovalis* (G. B. Sowerby I, 1833)	30		85				5				5						7.81
Family Veneridae																	
55. *Chione compta* (Broderip, 1835)		345									10	5					22.5
56. *Chione guatulcoensis* Hertlein and Strong, 1948	15	30	5							5							3.44
57. *Chionchione pulicaria* (Broderip, 1835)	30																1.875
58. *Cyclinella subquadrata* (Hanley, 1844)			5														0.31
59. *Dosinia dunkeri* (Philippi, 1844)			5														0.31
60. *Dosinia ponderosa* (Gray, 1838)			10				5										0.94
61. *Gouldia californica* Dall, 1917			460								60						32.5
62. *Lirophora kellettii* (Hinds, 1845)			10														0.625
63. *Lirophora mariae* (d'Orbigny, 1846)			5														0.31
64. *Megapitaria squalida* (G. B. Sowerby I, 1835)	5		460														29.06

Appendix 1. (continued)

Sampling station	52	24	47	35	23	48	26	51	34	18	22	30	33	50	25	29	Mean number of individuals per m^2
Type of substratum	MS	SS	MS	SS	SS	SC	SC	SC	SS	SS	SS	SC	SC	SC	SC	SC	
Depth (m)	18	24	40	48	49	53	57	60	66	71	72	74	83	84	99	112	
Temperature (°C)	28	30	24	16	26	29	26	25.5	17	15	14.5	16	16	19	14.5	14.5	
Dissolved oxygen (ml/l)	6.3	5.0	3.6	0.6	0.6	6.5	7.4	5.6	0.5	0.2	0.3	0.3	0.4	1.0	0.3	0.2	
65. *Periglypta multicostata* (G. B. Sowerby I, 1835)		5	5									5					0.94
66. *Pitar callicomatus* (Dall, 1902)			15			5											1.25
67. *Pitar concinnus* (G. B. Sowerby I, 1835)	60		10														4.375
68. *Pitar multispinosus* (G. B. Sowerby II, 1851)			5			5											0.625
69. *Transennella modesta* (G. B. Sowerby I, 1835)			20														1.25
Family Mactridae																	
70. *Mactrellona subalata* (Mörch, 1860)	10																0.625
71. *Mulinia pallida* (Broderip and G. B. Sowerby I, 1829)		10															0.625
Family Tellinidae																	
72. *Cymatoica undulata* (Hanley, 1844)			15	10			5			5	5						2.5
73. *Macoma elytrum* Keen, 1958			15				5										1.25
74. *Macoma siliqua* (C. B. Adams, 1852)										15	10	10					2.19
75. *Psammotreta aurora* (Hanley, 1844)			5														0.31
76. *Strigilla cicercula* (Philippi, 1846)		5	15														1.25
77. *Strigilla dichotoma* (Philippi, 1846)	55		5														0.31
78. *Strigilla interrupta* Mörch, 1860	30		110				5			5	5						9.69
79. *Strigilla* sp.			55														3.44
80. *Tellina carpenteri* Dall, 1900	30						5			10							2.81
81. *Tellina coani* Keen, 1971			200	10						20	5	5					15
82. *Tellina martinicensis* d'Orbigny, 1853			10							5		5					1.25
83. *Tellina pacifica* Dall, 1900			220														13.75
84. *Tellina pristiphora* Dall, 1900							10										0.625
Family Donacidae																	
85. *Donax gracilis* Hanley, 1845			75														4.69
Family Solecurtidae																	
86. *Tagelus politus* (Carpenter, 1857)			10														0.625
Family Semelidae																	
87. *Semele pallida* (G. B. Sowerby I, 1833)			15														0.94
88. *Semele verrucosa* Mörch, 1860			15														1.56
89. *Semelina campbellorum* Coan, 2003			285							5	60	90					27.5
Family Corbulidae																	
90. *Corbula ira* Dall, 1908															1210		75.625
91. *Corbula marmorata* Hinds, 1843			50														3.125
92. *Corbula nasuta* G. B. Sowerby I, 1833	25		2450	5	10			5			35	145					167.18
93. *Corbula ventricosa* A. Adams and Reeve, 1850				5								10	40				3.44
Family Pandoridae																	
94. *Pandora arcuata* G. B. Sowerby I, 1835	5							5									0.625
Family Verticordiidae																	
95. *Trigonulina novemcostatus* (A. Adams and Reeve, 1850)			25							5	10	55					5.94
Total individuals per station	97	134	2603	23	42	3	24	7	4	146	530	1117	24	17	414	11	
Total species per station	22	19	63	12	9	3	16	4	3	21	33	28	11	4	10	1	
Density (individuals/m^2) per station	485	670	13015	115	210	15	120	35	20	730	2650	5585	120	85	2070	55	

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A mature female of *Bathothauma* Chun, 1906 (Cephalopoda: Cranchiidae) from Hawaii

Janet R. Voight

Department of Zoology, The Field Museum of Natural History, 1400 S. Lake Shore Dr., Chicago, Illinois 60605, U.S.A., Jvoight@fieldmuseum.org

Abstract: A gravid female of the cranchiid squid genus *Bathothauma* Chun, 1906 was collected from 1027 m depth in excellent condition, other than having a ruptured ovary and tentacles reduced to stubs. The 2 mm diameter eggs, 18 spermatangia embedded in the skin of her mantle, head, and eye, and the very pronounced nidamental glands indicate full sexual maturity. The eggs are over three times larger than those previously reported in the genus.

Key words: Oegopsida, egg size, spermatangia, Taoniinae

The deep ocean forms the largest life-supporting area on earth, yet the animals that inhabit the area remain poorly known. Bathypelagic cephalopods are fairly abundant throughout the world's oceans (Clarke 1966) but continue to be scarce in collections. "Glass squids" of the Cranchiidae are a prime example. Many species of this group undergo ontogenetic vertical descent in which young stages of the life cycle occur at shallow depths; as the animals mature, they move deeper (Young 1978). This distributional pattern contributes to our limited knowledge of adults, and especially of reproductively mature members of this group.

Squids of the cranchiid genus *Bathothauma* Chun, 1906 have been collected circum-globally, at depths of 100 to nearly 2000 m (Voss 1980). Perhaps due to ontogenetic descent, the most familiar and frequently collected members of this genus are the remarkable young with unusually long stalks supporting eyes that extend well away from the axis of the body, and a very long brachial pillar that carries the arm crown and the mouth (Fig. 1). Individuals of up to at least 7 cm mantle length retain this larval morphology (Voss 1980) although the eyes and arms are thought to be become sessile in both sexes with maturation.

The most complete account of reproductive biology in *Bathothauma* is that of Aldred (1974). Of the 87 specimens considered, 3 specimens were maturing females, as evidenced by their detectable nidamental glands (Aldred 1974). Young (1978) also reported three gravid females trawled from near Oahu, Hawaiian Islands; he mentioned their enlarged nidamental glands and large eggs, and described spermatangia embedded in one of the females.

To advance our knowledge of the species-level diversity in the genus, this note reports data concerning a mature female collected from near 1000 m depth in Hawaiian waters. Voss *et al.* (1992) consider the genus *Bathothauma* to include four species and indicate that *Bathothauma lyromma* Chun, 1906, the only formally described species, is restricted to the Atlantic tropical and subtropical regions to about 45°N in the northeast Atlantic. As a result, only the generic identifier is applied to this specimen.

MATERIALS AND METHODS

The specimen was collected on 7 July 1996 in a modified opening/closing Tucker Trawl with a 10 m^2 mouth. Childress *et al.* (1978) describe the modifications to the trawl as including the addition of a 30-L thermally protecting cod-end to limit mechanical damage and heat shock to which the animals are exposed at recovery. The collection locality was off the island of Oahu at 21°35′00″N, 158°35′00″W to 21°20′00″N, 158°20′00″W at a maximum depth of 1027 m. When the female was removed from the cod-end, eggs began to spill out of her mantle cavity. She and her eggs were preserved in 8% formalin in buffered seawater for two weeks, then transferred to 70% ethanol at The Field Museum of Natural History (FMNH), Chicago, Illinois where she is catalogued in the Invertebrate collection as FMNH 286571.

Field Museum collections house six additional specimens of the genus *Bathothauma* that were collected by trawl from off Bermuda. Voss (1960) reported these specimens, identifying the largest two, with mantle lengths of 60 and 80 mm as females. These specimens are compared to the comparatively newly collected one from Hawaii.

Measurements were performed with electronic calipers. All eggs, whether loose or remaining in the mantle or remnants of the ovary, were removed and individually counted.

RESULTS

Externally, the female *Bathothauma* sp. from Hawaii appears to be in excellent condition (Fig. 2) although her

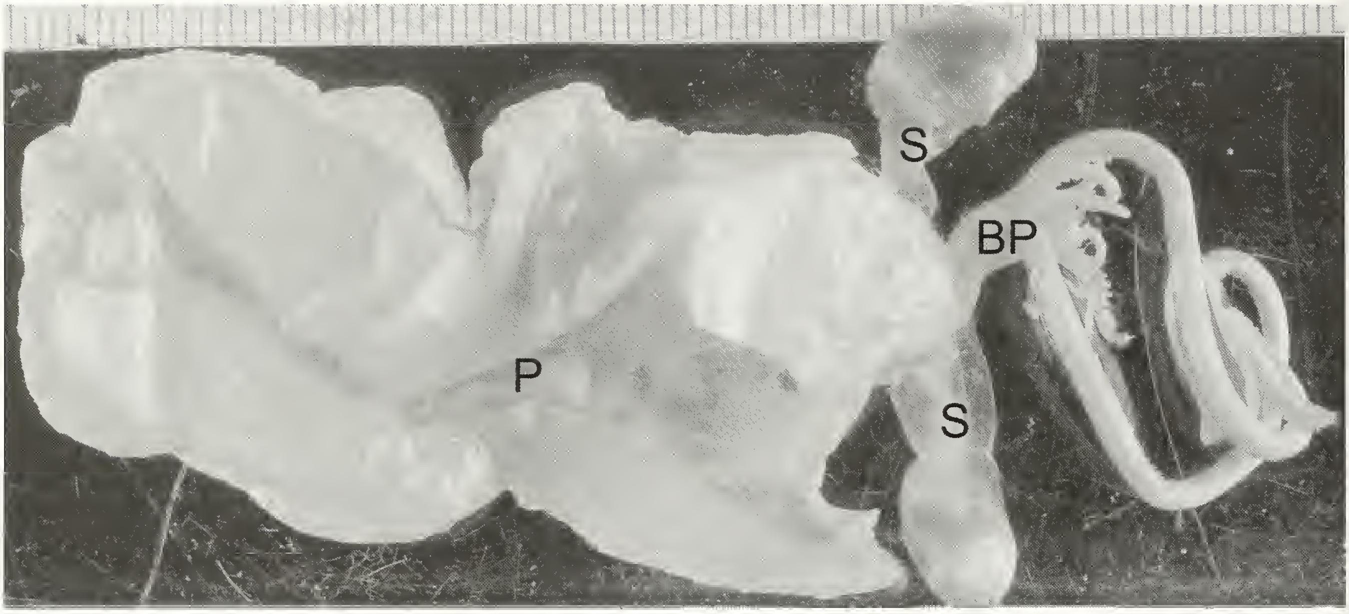

Figure 1. Ventral view of the male specimen of *Bathothauma lyromma* (FMNH 78328, mantle length 73 mm) collected from off Bermuda showing the larval morphology of the conspicuous stalks (S) carrying the eyes and the brachial pillar (BP). Note, however, the penis (P) inside the open mantle. The scale carries 1 mm increments. ©2008 The Field Museum, Z94484_01d, Photographer John Weinstein.

tentacles are represented only by stubs. The stubs appear to have been recently generated as the skin is puckered. Measurements are presented in Table 1. The arm tips are in excellent condition, with membranes on both sides; brachial end-organs and photophores are absent. Arm sucker size transitions fairly abruptly from large to small; the arm tips clearly carry suckers in two rows. Arm suckers carry only blunt teeth. The eyes are sessile on the sides of the head (Fig. 2).

Eight spermatangia lie just under the skin of the inner mantle on its right lateral wall. The ejaculatory apparatus emerges from the skin of the inner mantle and the skin on the external mantle is damaged in the area overlying the spermatangia. A total of six spermatangia are present medially on the left eye and on the neck posterior to the left eye. The dorsal mantle posterior to the right eye carries four. The mean length of four straight spermatangia is ~4.5 mm (range: 4.1 to 5.2).

When opened, in addition to a mass of loose amber-colored eggs, the female's mantle cavity contained a decapod shrimp (carapace length 6 mm) and three other small crustaceans. These may have contributed to the apparent rupture of the ovary. The total number of eggs is at least 1495, including minimally 747 that had been released prior to fixation, those removed from the mantle cavity and ovary, and those found in the jar. Each egg is undifferentiated and spherical (except where wrinkling of the outer cover implies preservation-caused shrinkage), with a diameter of 2 mm. Each egg appears to be identical in size. The outer cover of the ovary has scattered chromatophore organs; the remaining ovarian tissue carries small white sessile nodules that conceivably could be eggs or remnants of egg follicles. The oviducts generally lack any chromatophore organs and, although considerably enlarged, do not contain eggs. The oviducal glands are covered with a smattering of chromatophores and appear very similar in size and shape to the nidamental grants. The extremely large nidamental and oviducal glands are the most rigid organs in the mantle cavity (Fig. 2).

The two largest comparative specimens (FMNH 78328, FMNH 78329) were identified by Voss (1960) as females of *Bathothauma lyromma*. Although the specimens have juvenile characters of eyestalks and brachial pillar, they also have developing male reproductive organs. FMNH 78328 carries a 4.3 mm long penis (Fig. 1), but other male reproductive organs have been removed. Another large specimen from Bermuda (FMNH 78329) has a testis with two concentrated dots of purple pigment and accessory organs in the distal mantle that are visible to the unaided eye, and the penis was not removed from the associated tissue. There do not appear to be stored spermatophores. Neither individual shows clear evidence of hectocotylization.

DISCUSSION

The large eggs, presence of spermatangia, and enlarged,

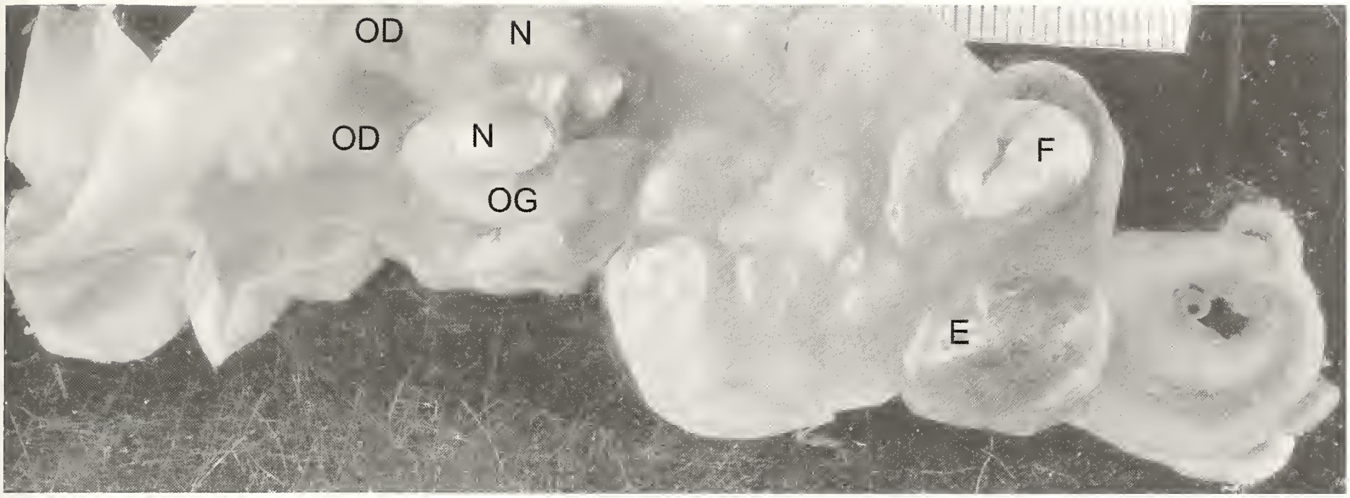

Figure 2. Ventral view of the female specimen of *Bathothauma* sp. (FMNH 286571, mantle length 93 mm). The funnel (F) is labeled, note the dark-colored eye (E) which carries two white spermatangia, the light-colored oviducts (OD) exposed in the mantle that emerge from the remnants of the ovary, and the conspicuous nidamental (N) and oviducal (OG) glands. The scale carries 1 mm increments. ©2008 The Field Museum, Z94485_01d, Photographer John Weinstein.

robust nidamental and oviducal glands (Fig. 2) all demonstrate that the female *Bathothauma* sp. examined was fully gravid. At 2 mm in diameter, the eggs of this female are over triple the 0.6 mm size of those in the only previous report for the genus (Aldred 1974). Unfortunately, the crustaceans that likely entered the mantle cavity of the female during collection preclude determination of how the eggs were held. The distribution of spermatangia is very much the same as Young (1978) reported in a female with very large eggs and nidamental glands.

The female reproductive anatomy seen here (Fig. 2) is very similar to that Chun (1910) reported for *Leachia* Lesueur, 1821. In *Leachia pacifica* (Issel, 1908), a member of the Cranchiinae, Young (1975) reported that the nidamental glands of near- or perhaps post-spawning females were gelatinous. Those of this female are robust with clear lamellae, with no indication of gelatinous texture. The contrast suggests that the females Young examined were post- rather than near spawning. The recovery of this female with intact arm tips also confirms that the brachial end organ is absent in this genus (Herring *et al.* 2002).

Graphically, none of the measurements or counts of this female distinguish it from the specimens represented on plots of three characters (Aldred 1974; fig. 1). These plots, however, rely on mantle length, a measurement that Voss *et al.* (1992) caution tends to be problematic as a size indicator in cranchiids due to preservation artifacts. The sexual maturation seen in the "larval" specimens collected off Bermuda imply that the change to adult morphology is not necessarily a pre-requisite for sexual development. The female specimen and data concerning her reproductive status are offered in hopes they aid futures studies of the genus and its species-level diversity.

Table 1. Measurements in mm and counts for *Bathothauma* sp. (FMNH 286571).

Mantle length	93
Head width (exclusive of eyes)	10
Head width (including eyes)	25
Arm length I	22.4
Arm length II	29.0
Arm length III	32.0
Number of arm suckers	57, 56
Arm length IV	29.0
Greatest eye length	18.9
Fin length at base	10.5
Maximum fin length	17.5
Nidamental gland length	14.6

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B. Seibel kindly invited my participation in the cruise that resulted in collection of the specimen; he and A. Lindgren provided helpful comments on the manuscript, as did two anonymous reviewers. The Captain and crew of the R/V *New Horizon* made the cruise successful. Marshall Field Fund of the Zoology Department at The Field Museum of Natural History supported my participation in the cruise.

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Conservation of the freshwater gastropods of Indiana: Historic and current distributions

Mark Pyron, Jayson Beugly, Erika Martin, and Matthew Spielman

Department of Biology, Ball State University, Muncie, Indiana 47306, U.S.A., mpyron@bsu.edu

Abstract: We surveyed Indiana collections of freshwater gastropods from 220 museum collection lots and found 39 species inhabiting Indiana historically. Collection dates of museum material ranged from 1900 to 2006, with a median date of 1986. We collected 17,593 gastropods at 123 sites, including 86 sites where museum material was previously collected. Our surveys were combined with recent literature surveys and indicate a total of 36 species are currently present in Indiana. The Indiana fauna is composed of three species that are apparently secure globally, and 36 species that are widespread, abundant, and globally secure, including two exotics. However, three species are locally extinct and many others are locally imperiled or vulnerable. The majority of freshwater gastropod taxa in Indiana are of local conservation concern. The causes of local gastropod extinctions are unknown but likely include agricultural impacts, hydrologic alterations from reservoirs, and pollution. We recommend thorough inventory, recognition, and protection of the aquatic gastropods in Indiana.

Key words: macroinvertebrates, endangered species, snails, distributions, biogeography

Freshwater gastropods inhabit all aquatic habitats in North America. However, relatively little information is available on species distributions and the ecological requirements of this group as a whole (Burch 1982, Thorp and Covich 2001, Stewart 2006). Although recent distributional studies exist for several states—*e.g.*, Iowa and New York (Jokinen 1992, Stewart 2006), large knowledge gaps remain for geographic distribution and species composition throughout much of North America. Aquatic gastropods are a large component of freshwater ecosystems, providing significant biomass as herbivores (Brown 2001, Brown *et al.* 2008). Freshwater gastropods are frequently used in water quality bioassessments because of the occurrence of several indicator species or groups that are sensitive to water quality and habitat alteration (Salanki *et al.* 2003). In addition, freshwater organisms are the most imperiled fauna in North America, and freshwater gastropods are a group that is at risk (Ricciardi and Rasmussen 1999, Brown *et al.* 2008).

Indiana's water resources are at risk because of a combination of agricultural, urban, and other human impacts. These effects result largely from patterns of human land use and subsequent effects on aquatic ecosystems (Allan 2004, Pyron *et al.* 2006). Although land use patterns are unlikely to change in the near future, identification and awareness of existing fauna can provide a baseline for further monitoring and conservation.

The earliest attempt to produce a guide to the aquatic gastropods of Indiana was by Goodrich and van der Schalie (1944), which included an identification key, habitat descriptions for each species, and brief descriptions of species distributions. However, most of the taxonomy used in this guide is out of date, and no other Indiana guides to aquatic gastropods have been published. Several more recent studies of Indiana aquatic gastropods provide presence/absence information for several taxa. Brown (1982) sampled aquatic gastropods of temporary ponds in northeast Indiana, to test for habitat overlap among species. He found six species that varied in abundances by pond type. Jokinen (2005) surveyed ponds of the Indiana Dunes National Park, to compare with a historic survey by Shelford (1913). Many of the ponds that Shelford (1913) surveyed have since been destroyed by industrial development. However, Jokinen (2005) found similar overall species richness for aquatic gastropods, due to a combination of species that appeared to be extinct and other species that were not found in Shelford's (1913) survey. Greenwood and Thorp (2001) studied the distributions and substrate selection of two caenogastropods in the Ohio River, upstream from Louisville, Kentucky. Both species, *Lithasia obovata* (Say, 1829) and *Pleurocera canaliculata* (Say, 1821), are large river specialists.

A current survey of Indiana snails is important because it provides information of local declines and extinctions that will require action from conservationists. Local extinctions may suggest problems with water quality or hydrologic alterations in the watershed, or other explanations for absence of gastropods at sites. Information on historical and current snail distributions throughout Indiana will thus be invaluable to future water quality managers and scientists. This study is such a survey of museum collections from Indiana, coupled with site visits to assess the current status of aquatic gastropods in the state.

MATERIALS AND METHODS

Study area

Indiana is in the mid-western United States, with physiography consisting primarily of glacial till plains (Visher 1922) and a total area of 94,000 km^2. The majority of the state is in the Central Lowland province with only local topographical relief. The southern limit of glaciation is a boundary line between the Central Lowland and the southern Low Plateau (~⅓ of the southern portion of the state). One fourth of the state along the north is in the Eastern Lake Section, with many moraine lakes formed from glacial drift. Two major watersheds drain the state: the Great Lakes are immediately north, and the remainder of the state is in the Mississippi River basin. The Illinois River watershed includes the Kankakee River to the northwest, the southern section of the state drains directly into the Ohio River, and the majority of the state is within the Wabash River watershed that drains to the Ohio River (Visher 1922).

The human footprint has been large in Indiana. About 98% of land is used for cropland, pasture, or development (GAP 1996). The northern 24% of the state was predominately wetland prior to European settlement, and 85% of these wetlands have been lost, with drainage for agriculture the primary cause (IDNR 1996). Water quality of Indiana streams was severely altered by humans (Gammon 1998). Nearly all Indiana streams that are within the Wabash River watershed (>70 % of the state) have hydrologic alterations (significant changes to the natural flow regime) caused primarily by agricultural effects and/or reservoir release (Pyron and Neumann 2008). The net result is a human-dominated landscape with habitat fragmentation and degradation, widespread pollution, and isolated plant and animal populations.

We used a two-step process for surveying aquatic gastropods of Indiana. The first step was to assess historical distributions using two natural history collections (Ohio State University Museum of Biological Diversity and University of Michigan Museum of Zoology) that contain aquatic gastropod material from Indiana. The second step was to return to sites where museum collections were taken and to re-survey the sites to determine if previously recorded species were still present. The modern surveys would also reveal any additional species not reported in earlier surveys. Museum visits included examining specimens and verifying identifications and recording location information and collection dates. No other Indiana guides to aquatic gastropods have been published, thus Burch's (1982) keys and notes are the primary reference for identification. Unless otherwise noted, nomenclatural taxonomy was from Turgeon *et al.* (1998) or Stewart (2006).

Field sampling

We visited 123 sites of which 86 were historical sites, in the summers of 2006-2008 to collect aquatic gastropods (Fig. 1). The additional sites were in locations where historic samples were sparse. Methods consisted of sampling all available habitats at each site, primarily by hand collections in shallow water, on woody debris, on the undersides of stones, and on aquatic vegetation. Deeper areas and fine substrates were sampled with a net. Collection durations were the equivalent of one individual searching for 60 min. For example, two persons searched for 30 min (Brown *et al.* 1998). Gastropods were preserved in 70% ethanol and identified in the laboratory to the lowest possible taxonomic level, using Burch (1982). All specimens will be deposited at the Illinois Natural History Survey. We determined global conservation status for species using The Nature Conservancy designations (www.natureserve.org), and we described the Indiana status based on our collections.

RESULTS

We found 220 lots of Indiana aquatic gastropods at the two museums, comprising 39 taxa at 86 sites. Museum ma-

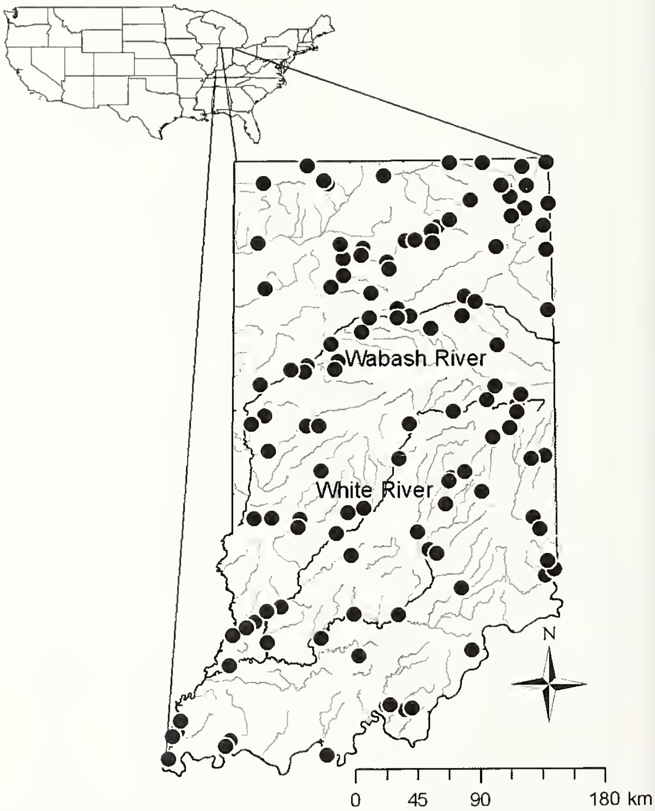

Figure 1. Sites sampled in 2006-2008. Coordinates are available from authors.

terial was collected between 1900 and 2006 with a median collection date of 1976. Our current survey of 123 sites yielded 17,593 individuals in 32 species (Figs. 2-17). Four additional taxa were included based on the survey by Jokinen 2005). Most taxa occurred at few sites—the average number of sites where an individual taxon occurred was 11 (range, 1-75; Figs. 2-17). The mean abundance of individuals collected at sites was 144 (range, 0-1463). The mean number of species per site was 3.3 (range, 0-8). Ten of the historic collection sites and five new sites had no gastropods. Seventeen of the 36 taxa we collected, or that were in Jokinen's (2005) collections, were not collected in previous surveys.

Mean water hardness was 300 mg $CaCO_3$/L and ranged from 40 to 1200. Mean water conductivity was 550 µmhos and ranged from 101 to 1800. Mean pH was 8.2 and ranged from 6 to 9.6. Although we found variation in mean water chemistry parameters among species, overall variation among sites was relatively low. The majority of species had mean hardness values of 300 mg $CaCO_3$/L, conductivity of 500 µmhos, and pH of 8.0. Only one species occurred at sites with an exceptional mean water chemistry value: *Ferrissia fragilis* (Tryon, 1863) was found at 18 sites with a mean pH of 7.2. We will examine the influence of environmental variables on gastropod assemblages in detail in a separate study.

The following list of taxa is organized by family. Distribution maps include historical sites from archival material and current (2006-2008) collections. Not all museum material included specific site details or dates. We did not include information on maps if collections lacked site information.

Family Valvatidae

Valvata bicarinata (Lea, 1841). Goodrich and van der Schalie (1944) reported the species occurred likely in every county in Indiana. This species has apparently declined or disappeared, as in Iowa (Stewart 2006). We consider it to be extinct in Indiana and secure in the rest of the range.

Valvata lewisi (Currier, 1868). No historical collections were found. The species was historically present in lakes in Kosciusko and Marshall County (Goodrich and van der Schalie 1944). We collected this species only at Clear Lake, Steuben County. The habitat was silt substrate and submerged vegetation. This species occurs in southern Canada from Quebec to British Columbia and northern U.S. from New York to Minnesota (Burch and Tottenham 1980). Jokinen (1992) found only one site for this species in New York, and Stewart (2006) determined the species is extinct in Iowa. We categorized it as critically imperiled in Indiana but it is secure in the rest of the range.

Valvata tricarinata (Say, 1817). No historical or recent collections were found. Jokinen (2005) found this species in a pond at Indiana Dunes National Lakeshore in 1992-1993. We categorized it as critically imperiled in Indiana but secure in other parts of the range.

Valvata sincera (Say, 1824). No historical or recent collections were found. Burch and Tottenham (1980) reported the range as Maine west to Alberta, and south to South Dakota and Indiana. We consider it to be extinct in Indiana but secure in other parts of the range.

Family Viviparidae

Viviparus georgianus (Lea, 1834). No historical collections were found. Wright (1932) collected this species in Indiana at four sites on Maxinkuckee Lake and its outlet, the Tippecanoe River. The species was historically present in the Wabash River and numerous Indiana lakes (Goodrich and van der Schalie 1944). We collected this species at two sites: Clear Lake (Steuben County) and Lake Wawasee (Elkart County). Both lakes had submerged vegetation and either sand or silt substrates. This species is distributed across the midwest and eastern U.S. (Burch and Tottenham 1980). Jokinen (1992) found many sites with this species in New York. It appears to be critically imperiled in Indiana but secure in other parts of the range.

Viviparus subpurpureus (Say, 1829). This species was collected historically at four sites that were large rivers and one pond (Fig. 2). We did not collect this species. The three large river sites have reservoirs within their watersheds, and reservoir releases likely produce hydrologic alterations to natural flow regimes. This species has a range through out the Mississippi River watershed to Iowa, Illinois, and Kentucky and south to Louisiana (Burch and Tottenham 1980, Brown *et al.* 1989). Goodrich and van der Schalie (1944) reported the species as confined to larger streams such as the Mississippi, Ohio, and Wabash rivers. Populations appear to be possibly extinct in Indiana but secure in other parts of the range.

Bellamya chinensis (Reeve, 1863). No historical collections were found. We collected this species at four sites that were lakes and rivers in the northern third of the state (Fig. 2). The sites had submerged macrophytes and various substrates. This Asian snail is an exotic species that has been introduced and subsequently dispersed across North America (Stewart 2006).

Bellamya japonica (von Martens, 1861). No historical collections were found. We collected this species at four sites that were lakes and rivers in the northern third of the state (Fig. 2). The sites had submerged macrophytes and various substrates. This Asian snail is an exotic species that has been introduced and subsequently dispersed across North America (Jokinen 1992).

Campeloma decisum (Say, 1817). We mapped all of the *Campeloma* spp. records together, following Stewart (2006). However, we recognized our current collections as *C. de-*

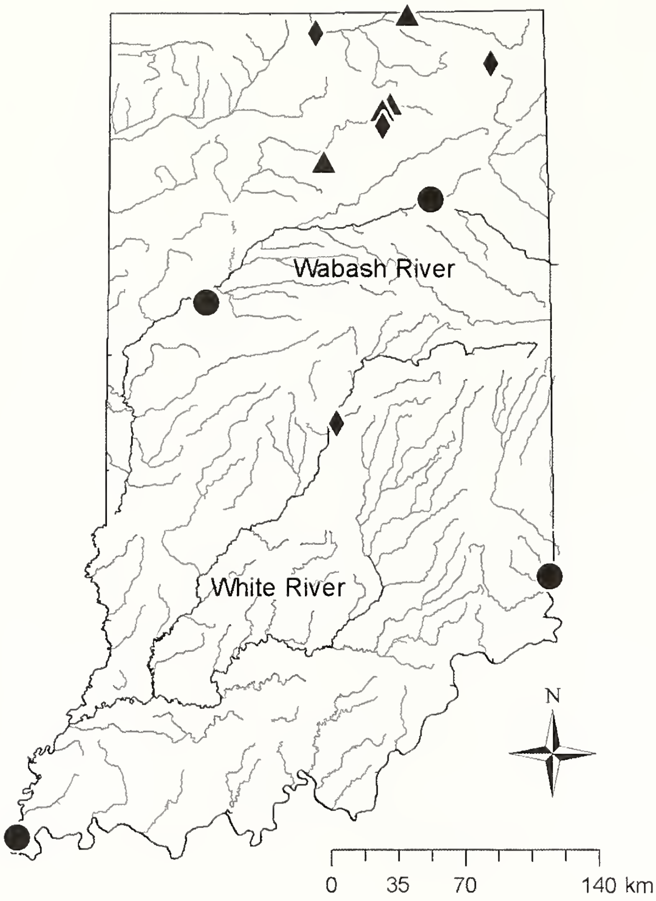

Figure 2. Distributions of historic *Viviparus subpurpureus* (circles), current *Bellamya chinensis* (triangles), and current *Bellamya japonica* (diamonds).

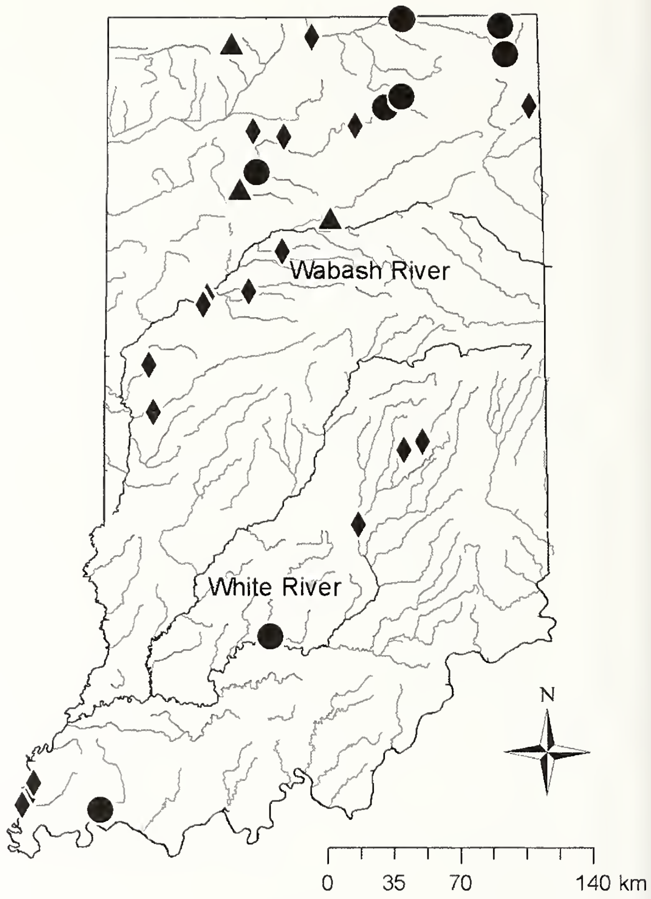

Figure 3. Distributions of historic (diamonds) and current *Campeloma decisum* (circles). Sites where historic and current collections occurred are triangles.

cisum. We found 20 historical collections and 11 current sites (Fig. 3). Habitats included macrophytes, woody debris, and various substrates of silt, sand, gravel, and cobble. *Campeloma* spp. occur in the Missouri and Mississippi watersheds (Stewart 2006 and references therein). They are common in lakes and rivers of Indiana and we classified them as secure.

Family Hydrobiidae

Birgella subglobosus (Say, 1825). No historical collections were found. We found the species at six sites (Fig. 4). Habitats included various substrate categories but lacked silt. The species was historically found throughout Indiana (Goodrich and van der Schalie 1944) with a range from Ohio west to Iowa, and from Michigan south to Alabama and Arkansas (Burch and Tottenham 1980). Jokinen (1992) collected the species in Lake Champlain, St. Lawrence watershed. We classified it as imperiled in Indiana but it is apparently secure in other parts of the range.

Cincinnatia integra (Say, 1821). We found one historical collection of this species from Lake James and we collected this species at one site on the Eel River. The habitat at the Eel River was gravel and sand substrates and submerged vegetation. Shelford (1913) found this species in ponds at the Indiana Dunes National Lakeshore. However, Jokinen (2005) did not find the species at the same ponds in 1992-1993. This species occurs in the Ohio River and tributaries in Ohio, Indiana, Kentucky, and southeastern Illinois (Burch and Tottenham 1980). Jokinen (1992) did not collect this species in New York, but it was found there historically. The species was historically common in Iowa (Stewart 2006). We classified it as critically imperiled in Indiana but secure in other parts of the range.

Pyrgulopsis lustrica (Pilsbry, 1890). Three historical collections were found: Tippecanoe Lake (Elkhart County), Pine Lake (La Porte County), and Lake Michigan (Lake County). We found the species in Little Turkey Lake and Big Turkey Lake (Steuben County). The habitats were sand or

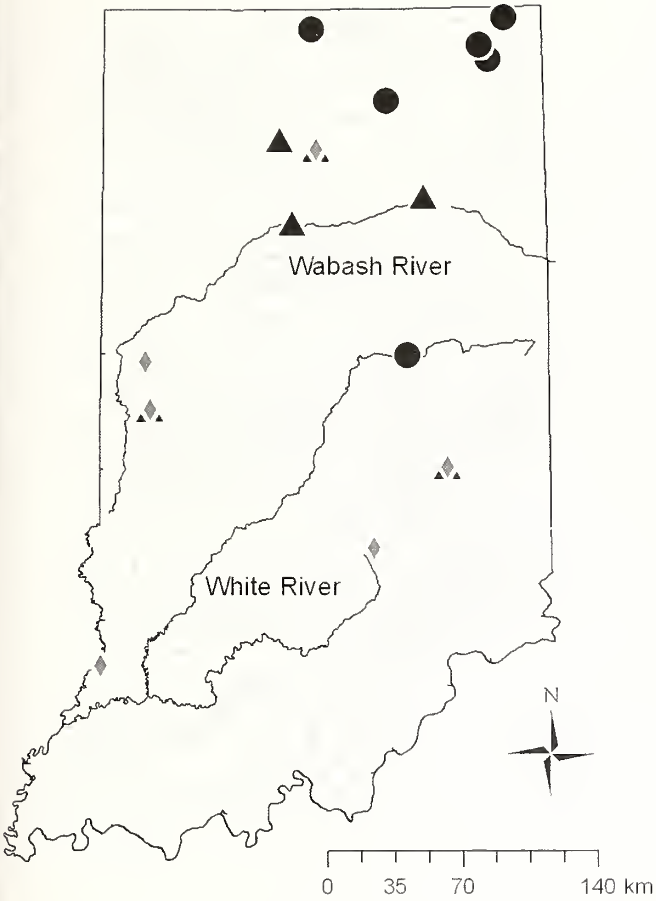

Figure 4. Distributions of current *Birgella subglobosus* (diamonds), current *Amnicola limosus* (circles), and *Pomatiopsis cincinnatiensis* (triangles).

silt substrates, woody debris, and emergent vegetation. This species occurs in southern Quebec and Ontario, and from Maine and New York west to Iowa and Minnesota (Burch and Tottenham 1980). Jokinen (1992) found this species at nine sites in New York. The species was found at several Iowa locations in 1979 (Stewart 2006). Goodrich and van der Schalie (1944) found this species was common in lakes, ponds, and streams that had heavy growths of macrophytes and algae. We classified its status as locally imperiled due to very few populations, but secure in other parts of the range.

Amnicola limosus (Say, 1817). No historical collections were found. We found the species at six sites (Fig. 4). Shelford (1913) found this species in ponds at the Indiana Dunes National Lakeshore. Jokinen (2005) found the species at one of the same ponds in 1992-1993. We found the species at six sites (Fig. 4). The species is imperiled in Indiana but secure in other parts of the range.

Family Pomatiopsidae

Pomatiopsis cincinnatiensis (I. Lea, 1850). This species is amphibious. No historical collections were found. We found the species at five sites (Fig. 4). The habitats were sand or silt substrates, and submerged or emergent vegetation. The river site had sand, gravel, and cobble substrates, and submergent vegetation. Goodrich and van der Schalie (1944) reported the historical distribution as Henry and La Porte Counties. The species is vulnerable in Indiana but it is apparently secure in other parts of the range.

Family Pleuroceridae

Elimia livescens (Menke, 1830). We included historical material that was misidentified as *Elimia semicarinata* (Say, 1829). We found 24 historical sites and 48 current sites (Fig. 5) of which five were lakes. The sites had various substrates including silt, sand, gravel, cobble, and boulders. This species occurs in the St. Lawrence River drainage from the Great

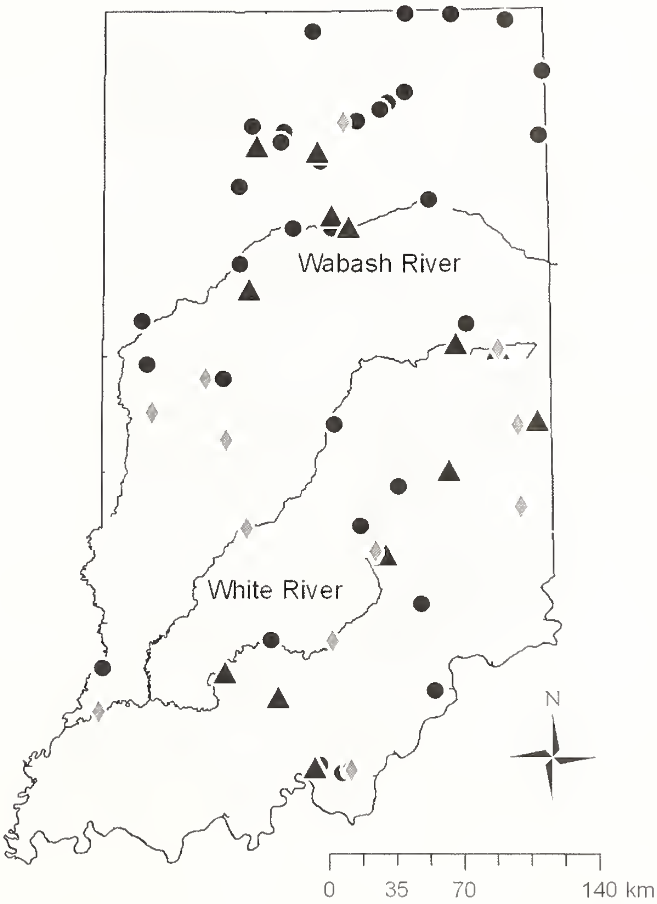

Figure 5. Distributions of historic (diamonds) and current *Elimia livescens* (circles). Sites where historic and current collections occurred are triangles.

Lakes to Lake Champlain and Quebec, east of the Scioto River in Ohio and west to the Illinois River (Burch and Tottenham 1980). The historic distribution was in the Wabash River watershed, the Maumee River watershed, the St. Joseph River watershed (Goodrich and van der Schalie 1944). The species was abundant in New York (Jokinen 1992). This is a common and abundant species in large rivers of Indiana with a secure status.

Pleurocera acuta (Rafinesque, 1831). We found 12 historical sites and 21 current sites for this species (Fig. 6) of which four were lakes. Habitat at the sites was varied with silt, sand, gravel, cobble, or boulder substrates and occasionally included macrophytes and/or woody debris. This species occurs in the Ohio River headwater streams and tributaries, the Great Lakes and tributaries, the Mississippi River watershed to Nebraska and Kansas, and the Cumberland and Duck rivers in Tennessee (Burch and Tottenham 1980). Goodrich and van der Schalie (1944) reported the historical distribution in Indiana as the upper Wabash River, tributaries, and lakes connected to the river, and the Maumee River and watershed. Stewart (2006) identified numerous collections in Iowa. Jokinen (1992) found the species at many New York locations. This species is common and abundant in Indiana with a secure status.

Pleurocera canaliculata (Say, 1821). We found 14 historical sites and two current sites on the Ohio River and White River (Fig. 7). Habitat at the Ohio River site was silt, sand, and riprap substrates with woody debris. The White River site had silt and riprap substrates. This species occurs in the Ohio River from Pittsburgh to Illinois, the Wabash River and its tributaries, aberrantly in the Tennessee River system, and to Omaha, Nebraska (Burch and Tottenham 1980). This species was found to be abundant in the Ohio River upstream from Louisville, Kentucky (Greenwood and Thorp 2001). Goodrich and van der Schalie (1944) listed the Indiana distribution as in the Wabash River above Lafayette downstream to the Ohio River, present in the White River

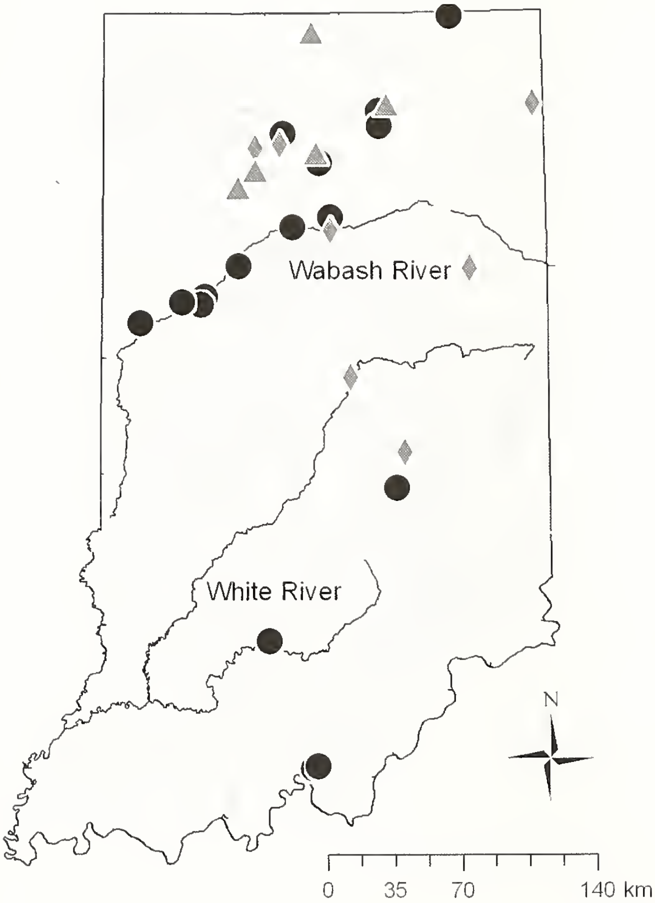

Figure 6. Distributions of historic (diamonds) and current *Pleurocera acuta* (circles). Sites where historic and current collections occurred are triangles.

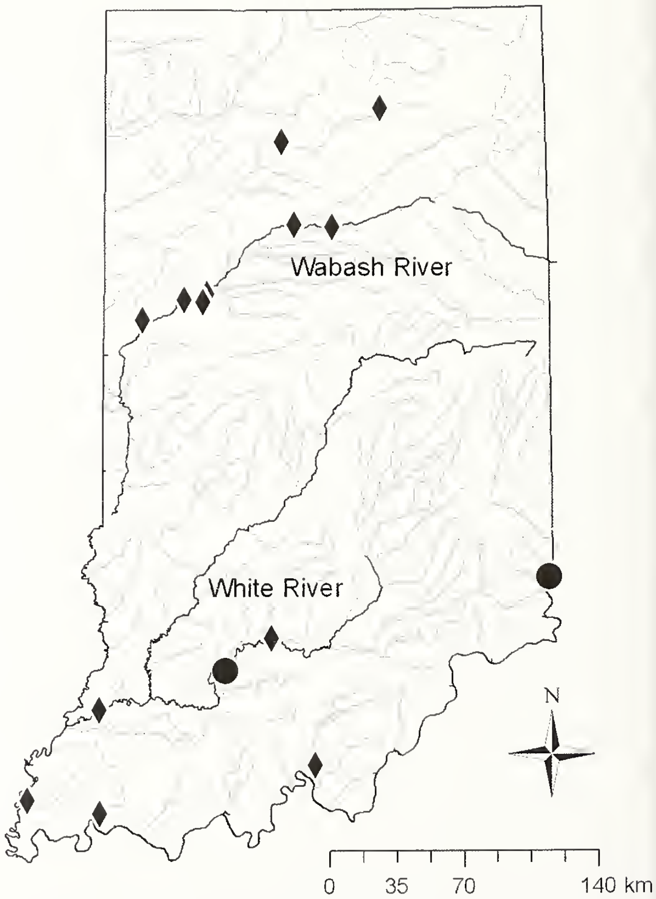

Figure 7. Distributions of historic (diamonds) and current *Pleurocera canaliculata* (circles).

and in the Ohio River. This is a rare species in Indiana but it is secure in other parts of the range.

Leptoxis praerosa (Say, 1821). No historical collections were found. We found the species at one site on the Blue River, Harrison County. The habitat was silt and riprap substrates with woody debris also present. Its range is the Ohio River below Cincinnati, Ohio to Elizabethtown, Illinois; the Cumberland River and tributaries; the Duck River, Tennessee; and the Tennessee River and tributaries (Burch and Tottenham 1980). Goodrich and van der Schalie (1944) reported the historical Indiana distribution as the Ohio River from Scioto County, Ohio to Pope County, Illinois, the Wabash River at Grand Chains, Posey County, and the Big Blue River, Crawford County. We categorized its status as critically imperiled in Indiana but it is secure in other parts of the range.

Lithasia obovata (Say, 1829). We found one historical collection from the Blue River in Harrison County, and one current site on the Eel River in Logansport. The habitat was silt, sand, and cobble substrates with emergent vegetation. This species was abundant in the Ohio River upstream from Louisville, Kentucky (Greenwood and Thorp 2001). This species occurs in the Ohio River and tributaries, in Pennsylvania, Ohio, Indiana, Illinois, Kentucky, and Tennessee (Burch and Tottenham 1980). Goodrich and van der Schalie (1944) reported this species present in the Wabash River downstream from Vincennes, in the Ohio River, the Big Blue River (Crawford County), and the Kentucky River in Kentucky. We categorized its status as critically imperiled in Indiana but it is apparently secure in other parts of the range.

Family Lymnaeidae

Fossaria spp. (Say, 1822). Stewart (2006) attributes many currently confused taxa to this group. We found seven historical collections that were in lakes and the Wabash River, and at 43 of our current sites (Fig. 8) of which five were lakes. Substrates at the sites varied with silt, sand, gravel, cobble, or riprap substrates, and occasional woody debris and vegetation present. Brown (1982) found this taxon in ponds at the Crooked Lake Field Station at Fort Wayne. Goodrich and van der Schalie (1944) reported this taxon present in ponds, lakes, and brooks in Kosciusko, Starke, Steuben, and La Porte Counties. Shelford (1913) found this taxon in ponds at the Indiana Dunes National Lakeshore. Jokinen (2005) found the taxon at the same ponds in 1992-1993. Burch and Tottenham (1980) described the range of taxa in this group to include eastern North America west to Vancouver Island. These taxa appear to be common and abundant in Indiana and thus secure.

Lymnaea stagnalis (Linnaeus, 1758). No historical collections were found. We found the species at Bass Lake, Starke County. The habitat of the site was silt and sand substrates and emergent vegetation present. This species range is the Great Lakes–St. Lawrence River drainage area northwest to the Mackenzie and Yukon River drainage areas, west to the Rocky Mountains, south to Colorado, and in Illinois and Ohio in the Mississippi drainage (Burch and Tottenham 1980). The historical Indiana distribution was small lakes and streams of the northern part of the state, and in Lake Michigan (Goodrich and van der Schalie 1944). We categorized its status as critically imperiled in Indiana but it is secure in other parts of the range.

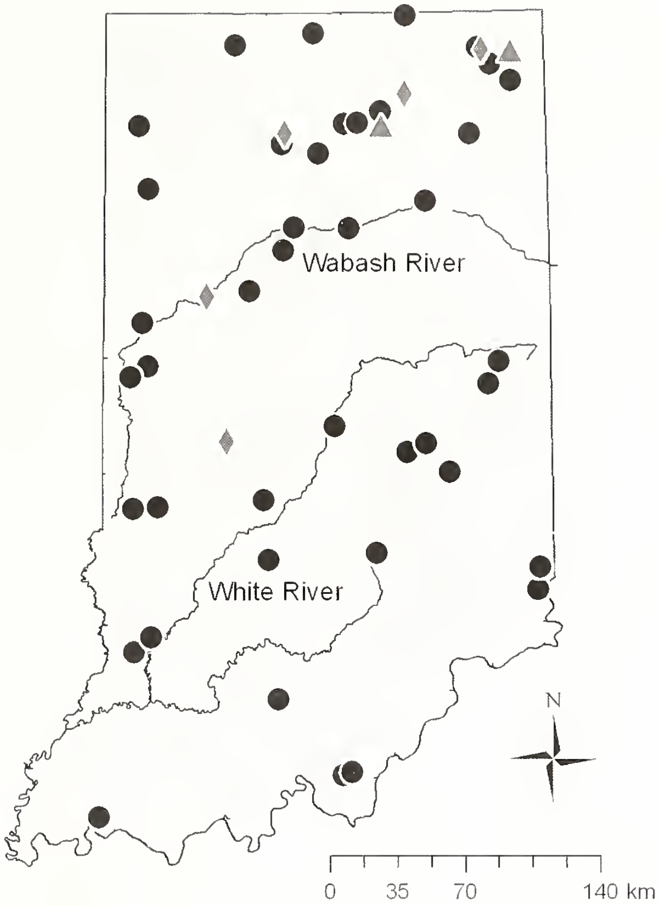

Figure 8. Distributions of historic (diamonds) and current *Fossaria* spp. (circles). Sites where historic and current collections occurred are triangles.

Stagnicola catascopium (Say, 1867). No historical collections were found. We found the species at one site, Fish Creek, Steuben County. The habitat of the site was silt and sand substrates and woody debris was present. Goodrich and van der Schalie (1944) reported the species was present in the Great Lakes and in other bodies of shallow water near Lake Michigan. The range is eastern Canada and Nova Scotia west to North Dakota, Great Slave Lake south to northern Iowa, northern Ohio, and Maryland (Burch and Tottenham 1980). We categorized its status as critically imperiled in Indiana but it is secure in other parts of the range.

Stagnicola caperata (Say, 1829). One historical collection was found from the Maumee River. We did not find this species. Goodrich and van der Schalie (1944) suggested the species occurred in every county of Indiana. The range is Quebec and Massachusetts west to California, Yukon Bay, and James Bay south to Maryland, Indiana, Colorado, and California (Burch and Tottenham 1980). We categorized its status as critically imperiled in Indiana but it is secure in other parts of the range.

Stagnicola elodes (Say, 1821). No historical collections were found. We found this species at 17 sites (Fig. 9) of which four were lakes. The habitats varied with substrates of silt, sand, gravel, cobble, or hardpan and woody debris and/or vegetation occasionally present. Goodrich and van der Schalie (1944) reported this species was expected in ditches, ponds, and shallow parts of lakes with heavy vegetation. Brown (1982) found this species was abundant in ponds at the Crooked Lake Field Station at Fort Wayne. Shelford (1913) found this species in ponds at the Indiana Dunes National Lakeshore. Jokinen (2005) found the species at the same ponds in 1992-1993. It is common and abundant in Indiana and is secure.

Stagnicola exilis (I. Lea, 1838). No historical collections were found. We found this species at Brown Ditch, Newton County. The habitat was silt substrate with submerged macrophytes. Goodrich and van der Schalie (1944) found this species to occur in temporary aquatic habitats. We categorized its status as critically imperiled in Indiana but it is secure in other parts of the range.

Pseudosuccinea columella (Say, 1817). No historical collections were found. We found 16 current stream sites (Fig. 10) of which seven were lakes or ponds. Habitats varied with substrates of silt, sand, gravel, or cobble and woody debris and/or vegetation occasionally present. The range is eastern North America generally west to Minnesota and eastern Kansas, south to central Texas and Florida (Burch and Tottenham 1980). Goodrich and van der Schalie (1944) re-

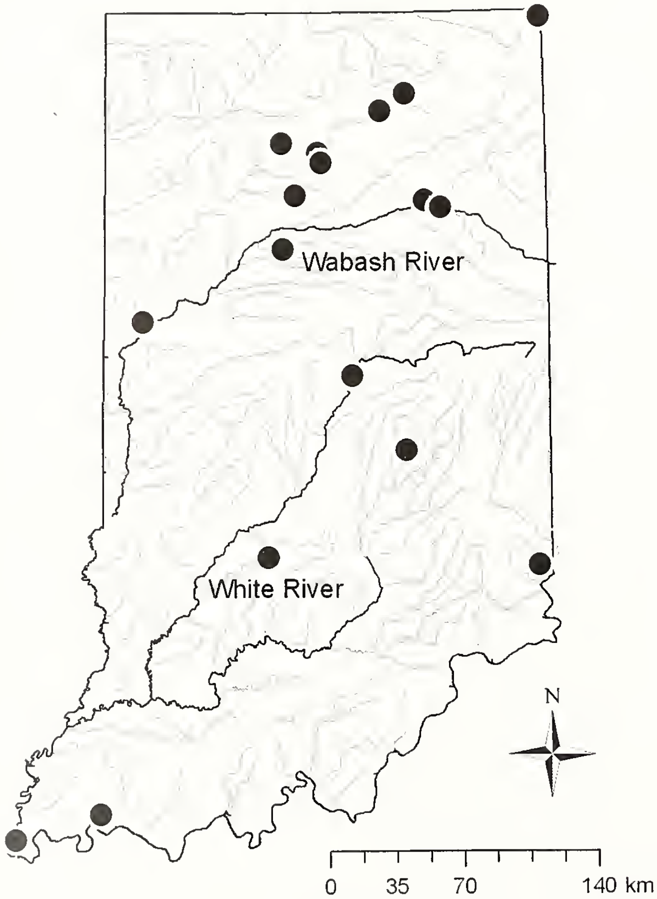

Figure 9. Distribution of current *Stagnicola elodes.*

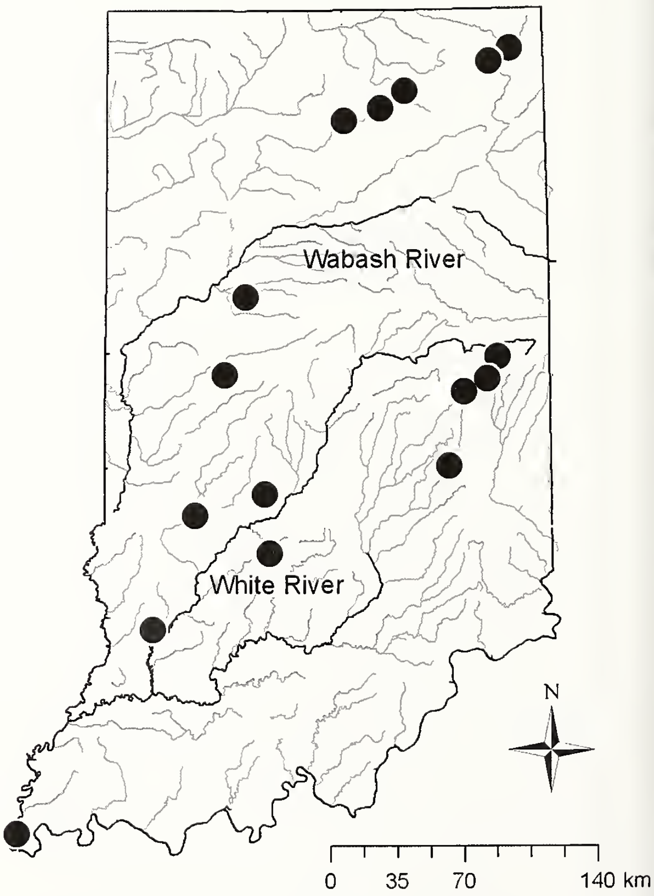

Figure 10. Distribution of current *Pseudosuccinea columella.*

ported this species present in northern Indiana counties. The species is common in New York (Jokinen 1992). It appears to be common in Indiana and is secure.

Family Physidae

Physella gyrina (Say, 1821). We found 22 historical sites and eight current sites (Fig. 11) of which one was a lake. Habitats varied with silt, sand, gravel, or cobble substrates and woody debris and/or vegetation present. Brown (1982) found this species was abundant in ponds at the Crooked Lake Field Station at Fort Wayne. Shelford (1913) found this species in ponds at the Indiana Dunes National Lakeshore. Jokinen (2005) found the species at the same ponds in 1992-1993. The species appears to be common and abundant and is secure.

Physella acuta (Draparnaud, 1805). We found 17 historical sites and 94 current sites (Fig. 12). Habitats varied with substrates of silt, sand, gravel, riprap, or cobble and woody debris and/or vegetation occasionally present. This species occurs throughout North America (Burch and Tottenham 1980) and is abundant in Indiana. We consider it to be secure in Indiana.

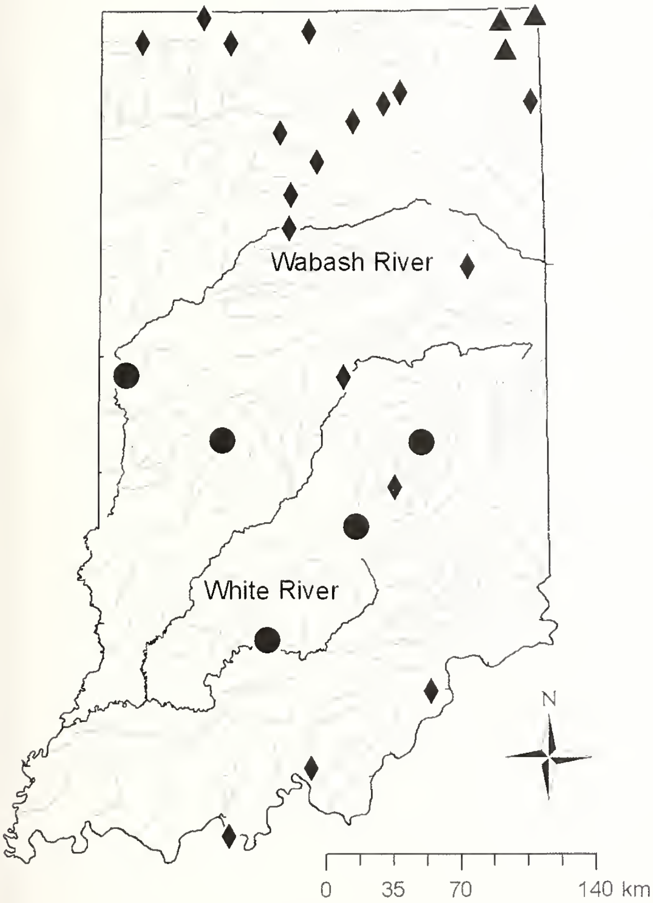

Figure 11. Distributions of historic (diamonds) and current *Physella gyrina* (circles). Sites where historic and current collections occurred are triangles.

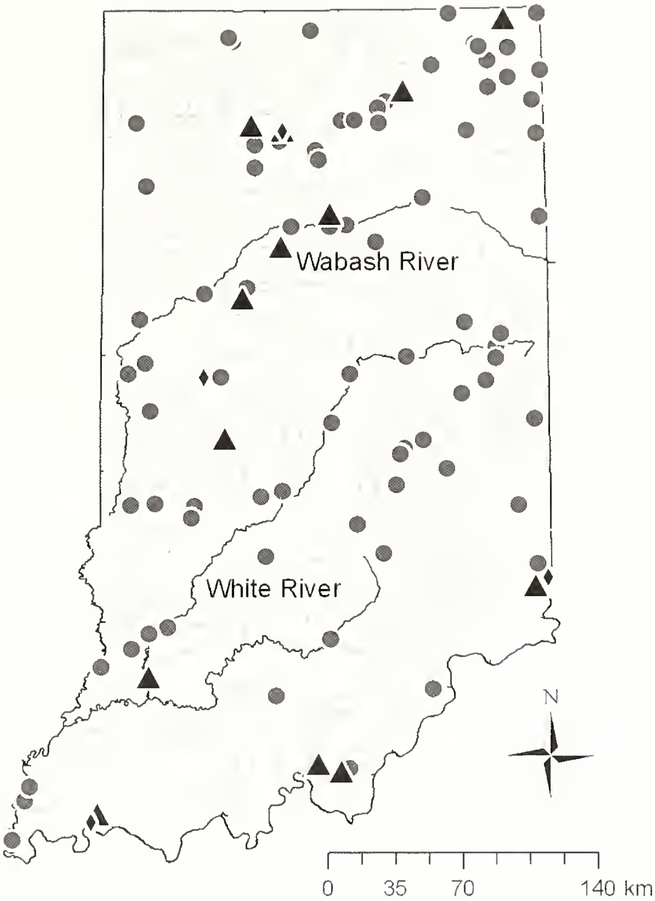

Figure 12. Distributions of historic (diamonds) and current *Physella acuta* (circles). Sites where historic and current collections occurred are triangles.

Aplexa elongata (Say, 1821). Two historical collections were found: Tippecanoe Lake (Elkhart County) and the Elkhart River (Noble County). We did not find this species. Brown (1982) found this species was abundant in ponds at the Crooked Lake Field Station at Fort Wayne. Jokinen (2005) found the species in temporary aquatic habitats in the Indiana Dunes National Seashore in 1992-1993. Its range is Ontario to Saskatchewan, Canada, and Alaska (Burch and Tottenham 1980). We categorized its status as imperiled in Indiana but it is secure in other parts of the range.

Family Planorbidae

Gyraulus circumstriatus (Tryon, 1866). We found one historical collection from Rock Creek, Carroll County, and none in our collections. Goodrich and van der Schalie (1944) reported the species present in Lake James, Lake Maximkuckee, and Webster Lakes in northern Indiana. This species occurs from Connecticut north to Quebec, west to Alberta, and south in the Rocky Mountains to New Mexico (Burch and Tottenham 1980). Jokinen (1992) found the species in New York collections but commented that it appears to be intolerant to low pH and low calcium, as are most snails. The species appears to be extinct in Indiana but secure in other parts of the range.

Gyraulus deflectus (Say, 1824). No historical collections were found although Shelford (1913) found this species in ponds at the Indiana Dunes National Lakeshore. Jokinen (2005) did not find them during 1992-1993 surveys of the same ponds. We found the species at 10 sites (Fig. 13). Habitats varied with substrates of silt, sand, gravel, or cobble and woody debris and/or vegetation occasionally present. We consider the species to be vulnerable in Indiana but it is secure in other parts of the range.

Gyraulus parvus (Say, 1817). We found nine historical sites and nine current sites (Fig. 14). Habitats varied with substrates of silt, sand, gravel, cobble, or boulder and woody debris and/or vegetation occasionally present. Shelford (1913) found this species in ponds at the Indiana Dunes National Lakeshore. Jokinen (2005) found the species at the same ponds in 1992-1993. Brown (1982) found this species was abundant in ponds at the Crooked Lake Field Station at Fort Wayne. Its range is all of North America (Burch and Tottenham 1980). Jokinen (1992) found the species at many sites in New York. Goodrich and van der Schalie (1944) comment that the species was "doubtless present in every county." The species appears to be common and abundant and is secure.

Helisoma anceps (Menke, 1830). No historical collections were found. Shelford (1913) found this species in ponds at the Indiana Dunes National Lakeshore. Jokinen (2005) did not found the species at the same ponds in 1992-1993. We found six current sites in lakes and streams (Fig.

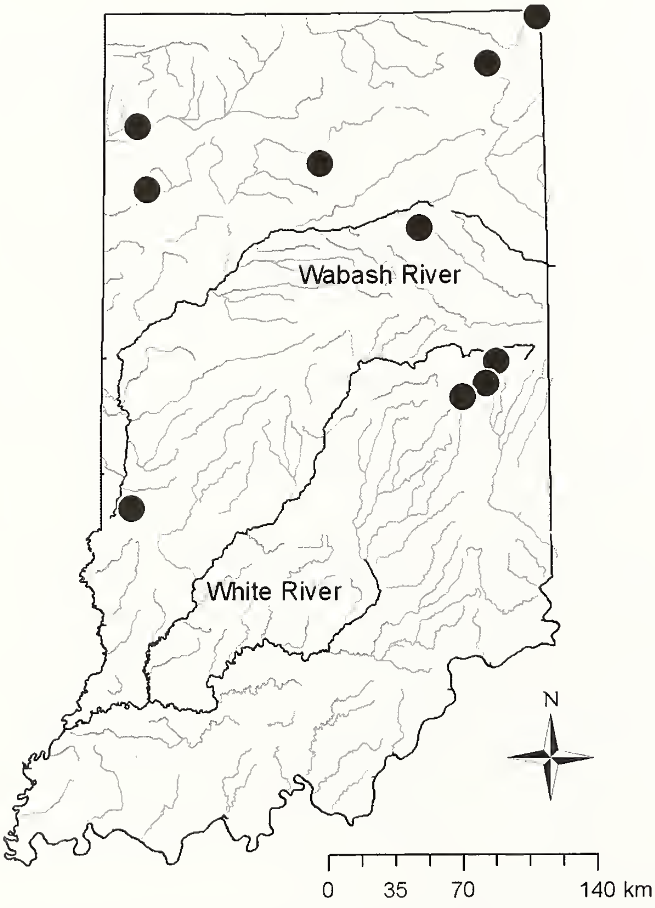

Figure 13. Distribution of current *Gyraulus deflectus.*

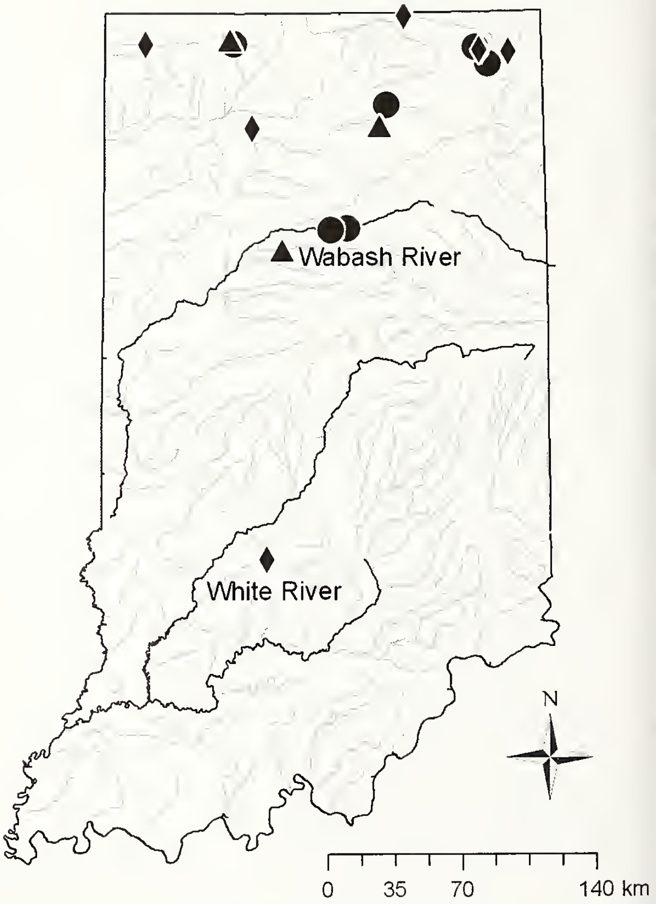

Figure 14. Distributions of historic (diamonds) and current *Gyraulus parvus* (circles). Sites where historic and current collections occurred are triangles.

15). Habitats varied with substrates of silt, sand, gravel, or cobble and vegetation occasionally present. The range is throughout North America from James and Hudson Bays south to Georgia, Alabama, Texas, and northwestern Mexico, west to southwestern Northwest Territories (Burch and Tottenham 1980). The species is widespread across New York (Jokinen 1992). Goodrich and van der Schalie (1944) reported the species was probably in every part of Indiana. We consider the species to be imperiled in Indiana but it is secure in parts of the range.

Planorbella campanulata (Say, 1821). No historical collections were found. We found one current site in Lake Wawasee. The habitat was sand and riprap substrate and submergent macrophytes. Shelford (1913) found this species in ponds at the Indiana Dunes National Lakeshore. Jokinen (2005) did not find the species at the same ponds in 1992-1993. The range is Vermont west to North Dakota, south to Ohio and Illinois, northward to Great Slave Lake (Burch and Tottenham 1980). The species was common in New York (Jokinen 1992). Goodrich and van der Schalie (1944) report that the species is most likely limited to the lakes area of Indiana, and the species is intolerant of domestic sewage. We consider the species to be imperiled in Indiana but it is secure in parts of the range.

Planorbella trivolvis (Say, 1817). Two historical collections were found in Half Moon Pond and Bass Lake. We found it at 16 sites (Fig. 16) of which five were lakes. Habitats varied with substrates of silt, sand, gravel, cobble, or boulder and woody debris and/or vegetation occasionally present. The species was not found at either historical site. Shelford (1913) found this species in ponds at the Indiana Dunes National Lakeshore. Jokinen (2005) found the species at the same ponds in 1992-1993. Brown (1982) found this species was abundant in ponds at the Crooked Lake Field Station at Fort Wayne. The range is Atlantic coast and Mississippi River drainages, northward to Arctic Canada and Alaska, and southward to Tennessee and Missouri (Burch and Tottenham 1980). The species is widespread and abundant in New York (Jokinen 1992). The species was histori-

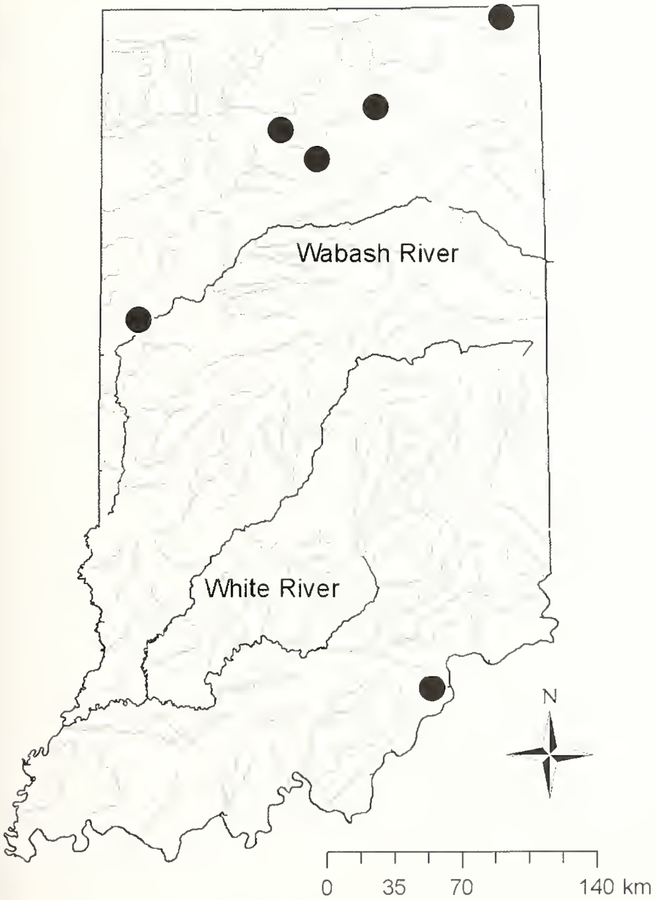

Figure 15. Distribution of current *Helisoma anceps.*

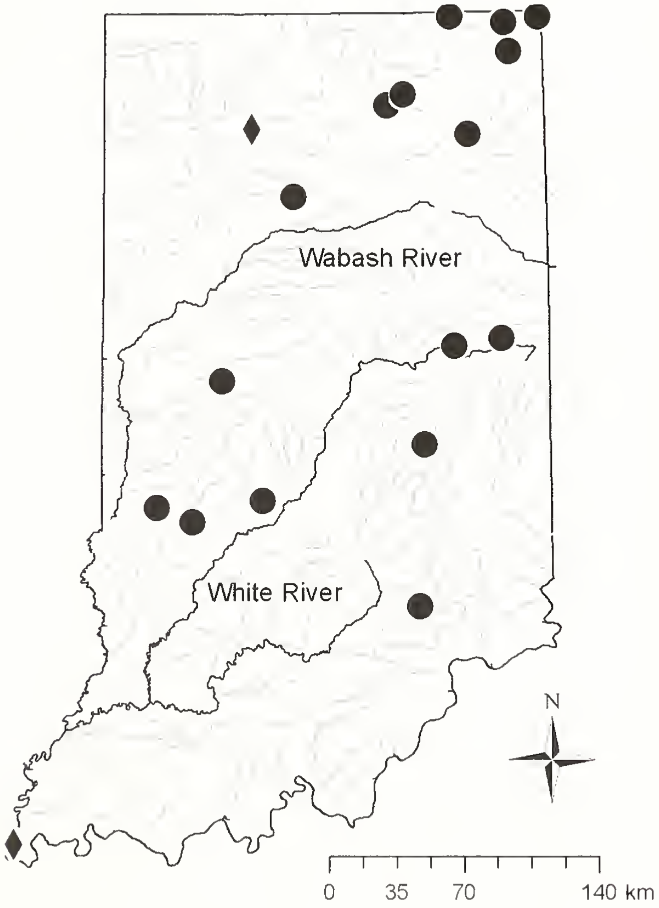

Figure 16. Distributions of historic (diamonds) and current *Planorbella trivolvis* (circles).

cally common in lakes and likely present throughout Indiana (Goodrich and van der Schalie 1944). We categorized the status of this species as locally and globally secure.

Planorbula armigera (Say, 1821). No historical collections were found and we did not find them in our collections. However, Shelford (1913) found this species in ponds at the Indiana Dunes National Lakeshore. Jokinen (2005) found the species at the same ponds in 1992-1993. Historical locations in Indiana were lakes in Lake, La Porte, Steuben, Marshall, and Kosciusko Counties (Goodrich and van der Schalie 1944). We categorized its status as critically imperiled in Indiana but it is secure in other parts of the range.

Promenetus exacuous (Say, 1821). No historical collections were found. We found the species at two lakes in Steuben County, Clear Lake and Pleasant Lake. Habitats were silt substrates with submerged vegetation at Clear Lake, and silt and sand substrates with emergent vegetation at Pleasant Lake. Shelford (1913) found this species in two ponds at the Indiana Dunes National Lakeshore. Jokinen (2005) found the species at one of the ponds in 1992-1993. The species was assumed to occur throughout Indiana by Goodrich and van der Schalie (1944). We categorized its status as critically imperiled in Indiana but it is secure in other parts of the range.

Family Ancylidae

Ferrissia fragilis (Tryon, 1863). No historical collections were found. We found three current stream sites: Clear Creek (Huntington County), Coal Creek (Fountan County), and the Tippecanoe River (Kosciusko County). Habitats varied with substrates of silt, sand, gravel, cobble, or boulder and woody debris occasionally present. The range is New York to Michigan, California, and Texas (Burch and Tottenham 1980). Stewart (2006) found that the species has not been observed in Iowa since 1912. However, he mentioned that the species is tiny and easily overlooked. In New York, the species is fairly common (Jokinen 1992). The historical locations that were published in Indiana were Clear Lake and a pond in La Porte County (Goodrich and van der Schalie 1944). We categorized its status as imperiled in Indiana but it is secure in other parts of the range.

Ferrissia parallelus (Haldeman, 1841). No historical collections were found and we did not collect this species. Shelford (1913) found this species in ponds at the Indiana Dunes National Lakeshore. Jokinen (2005) also found the species in ponds at the Indiana Dunes National Lakeshore in 1992-1993. The only other historical location in Indiana was Lake Maxinkuckee (Goodrich and van der Schalie 1944). The species appears to be extinct in Iowa (Stewart 2006) and rare in Indiana. We categorized its status as critically imperiled in Indiana but it is secure in other parts of the range.

Ferrissia rivularis (Say, 1817). No historical collections were found. We found 18 current sites (Fig. 17) of which four were lakes. Habitats varied with substrates of silt, sand, gravel, cobble, or boulder and woody debris and/or vegetation occasionally present. The range is most of North America: northward into the Hudson Bay lowlands and northwestward to Saskatchewan, south to North Carolina and New Mexico, west to California and Oregon (Burch and Tottenham 1980). The species is fairly common in New York (Jokinen 1992). The historical distribution in Indiana was Lake Knox and Henry Counties (Goodrich and van der Schalie 1944). The species appears to be widely distributed in Indiana, except that it is easily overlooked. It is common, widespread, and secure.

Laevapex fuscus (C. B. Adams, 1841). No historical collections were found. We found one current site, the Eel River at Logansport. The habitat was silt and riprap substrates and woody debris was present. Jokinen (2005) found the species in one pond at the Indiana Dunes National Lakeshore in 1992-1993. Historical locations in Indiana were lakes in Marshall and La Porte Counties, and Grassy Creek, Kosci-

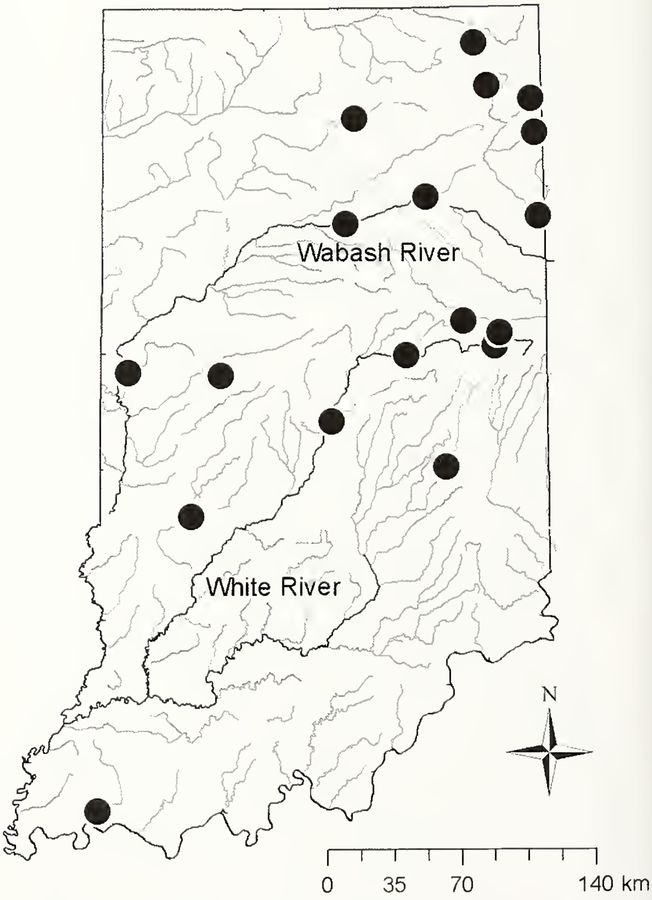

Figure 17. Distribution of current *Ferrissia rivularis*.

Table 1. Summary of aquatic gastropods of Indiana by current taxa (from Stewart 2006), synonyms from Goodrich and van der Schalie (1944), historic sites based on museum material, current sites where we collected the species, and observation status using The Nature Conservance global ranking system (www.natureserve.org). GS refers to species that are presumed extinct, GH are possibly extinct, G1 are critically imperiled, G2 are imperiled, G3 are vulnerable, G4 are apparently secure, and G5 are secure.

Current taxa	Synonyms	Historic sites	Current sites	Conservation status
Valvatidae				
Valvata bicarinata (Lea, 1841)		0	0	G5
Valvata lewisi (Currier 1868)	*Valvata lewisii*	0	1	G5
Valvata tricarinata (Say, 1817)		0	0[a]	G5
Valvata sincera (Say, 1824)		0	0	G5
Viviparidae				
Viviparus georgianus (Lea, 1834)	*Valvata contectoides*	0	2	G5
Viviparus subpurpureus (Say, 1829)		4	0	G5
Bellamya chinensis (Reeve, 1863)		0	4	Exotic
Bellamya japonica (von Martens, 1861)		0	4	Exotic
Campeloma decisum (Say, 1817)	*Campeloma* spp.	20	11	G5
Hydrobiidae				
Birgella subglobosus (Say, 1825)	*Somatogyrus subglobosus*	0	6	G4
Cincinnatia integra (Say, 1821)		1	1	G5
Pyrogulopsis lustrica (Pilsbry, 1890)	*Amnicola lustrica*	3	2	G5
Amnicola limosus (Say, 1817)	*Amnicola limosa, Amnicola parva*	0	6[a]	G5
Pomatiopsidae				
Pomatiopsis cincinnatiensis (I. Lea, 1850)		0	6	G4
Pleuroceridae				
Elimia livescens (Menke, 1830)	*Goniobasis livescens*	24	48	G5
Pleurocera acuta (Rafinesque, 1831)		12	21	G5
Pleurocera canaliculata (Say, 1821)		14	2	G5
Leptoxis praerosa (Say, 1821)	*Anculosa praerosa*	0	1	G5
Lithasia obovata (Say, 1820)		1	1[b]	G4
Lymnaeidae				
Fossaria spp. (Say, 1822)	*Lymnaea humilis, Lymnaea dalli, Lymnaea parva*	7	43[a]	G5
Lymnaea stagnalis (Linnaeus, 1758)		0	1	G5
Stagnicola catascopium (Say, 1867)	*Lymnaea catascopium*	0	1	G5
Stagnicola caperata (Say, 1829)	*Lymnaea caperata*	1	0	G5
Stagnicola elodes (Say, 1821)	*Lymnaea palustris, Lymnaea reflexa*	0	17[a]	G5
Stagnicola exilis (I. Lea, 1838)	*Lymnaea exilis*	0	1	G5
Pseudosuccinea columella (Say, 1821)	*Lymnaea columella*	0	16	G5
Physidae				
Physella gyrina (Say, 1821)	*Physella heterostropha, Physella sayii, Physella ancillaria*	22	8[a]	G5
Physella acuta (Draparnaud, 1805)	*Physella heterostropha, Physella integra, Physella walkeri*	17	94	G5
Aplexa elongata (Say, 1821)	*Aplexa hypnorum*	2	0[a]	G5
Planorbidae				
Gyraulus circumstriatus (Tryon, 1866)		1	0	G5
Gyraulus deflectus (Say, 1824)	*Gyraulus hirsutus*	0	10	G5
Gyraulus parvus (Say, 1817)		9	9[a]	G5
Helisoma anceps (Menke, 1830)	*Helisoma antrosum*	0	6	G5
Planorbella campanulata (Say, 1821)	*Helisoma campanulatum*	0	1	G5
Planorbella trivolvis (Say, 1817)	*Helisoma trivolvis*	2	16[a]	G5
Planorbula armigera (Say, 1821)		0	0[a]	G5
Promenetus exacuous (Say, 1821)	*Menetus exacuous*	0	2[a]	G5
Ancylidae				
Ferrissia fragilis (Tryon, 1863)	*Gundlachia meekiana*	0	3	G5
Ferrissia parallelus (Haldeman, 1841)	*Ferrissia parallela*	0	0[a]	G5
Ferrissia rivularis (Say, 1817)	*Ferrissia tarda*	0	18	G5
Laevapex fuscus (C. B. Adams, 1841)	*Ferrissia fusca*	0	1[a]	G5

[a] Refers to taxa that were collected in Indiana by Jokinen (2005). [b] Refers to taxa that were collected on the Ohio River main stem by Greenwood and Thorp (2001).

usko County (Goodrich and van der Schalie 1944). We categorized its status as critically imperiled in Indiana but it is secure in other parts of the range.

DISCUSSION

Our historical surveys of museum records and literature search indicated 39 species present historically in Indiana. Our 2006-2008 survey and literature search resulted in 36 species of aquatic gastropods, including two exotics. Three species are apparently locally extinct in the state (*Valvata bicarinata, Valvata sincera,* and *Gyraulus circumstriatus*) but globally secure. Our species richness estimates are similar to species estimates for other states (Stewart 2006). For example, states with published aquatic gastropod species richness values are: Connecticut (35 species; Jokinen 1983), Maine (45 species; Martin 1999), New York (61 species; Jokinen 1992), Virginia (53 species; Stewart and Dillon 2004), Kentucky (29 species; Branson *et al.* 1987), and Iowa (49 species; Stewart 2006). The conservation status of Indiana's gastropods is: three taxa that are apparently secure globally and 36 taxa that are widespread, abundant, and globally secure, including two exotics (Table 1). However, three taxa are locally extinct and many others appear locally imperiled or vulnerable.

Of three Indiana species presumed to be locally extinct, only *Valvata bicarinata* was collected in Indiana by Goodrich and van der Schalie (1944) and it appears to be declining elsewhere (Stewart 2006). Whether *Valvata sincera* was ever collected in Indiana is an open question. *Gyraulus circumstriatus* is likely locally extinct due to water quality degradation, as it is intolerant to low pH and calcium (Jokinen 1992).

Only 31 of the sites where the museum material was collected had the same species present in our collections at those sites. Our interpretation is that these species are likely no longer present at the majority of historic sites. Our collection technique may have missed individuals, but the probability of missing species that were historically abundant seems unlikely. Explanations for local species extinctions include extensive habitat degradation throughout the state from agricultural impacts, hydrologic alteration by reservoirs, and pollution. The majority of Indiana watersheds have hydrologic alterations due to reservoir release and/or channelization for agriculture drainage (Pyron and Neumann 2008). Watersheds that are upstream from reservoirs are also negatively impacted by the reservoir (Pringle 1997).

Conservation of aquatic gastropods should be considered as important as conservation of other aquatic organisms, if only for preservation of phylogenetic diversity. In Alabama, 65% of gill-breathing endemic freshwater snails are extinct, endangered, threatened, or of special concern (Lydeard and Mayden 1995). Although the southeastern U.S. has the highest diversity of freshwater organisms on the continent, all freshwater fauna of North America are facing similar losses of biodiversity (Ricciardi and Rasmussen 1999). Ricciardi and Rasmussen (1999) projected future extinction rates for freshwater organisms in North America at 4% per decade. This is a similar depletion rate as for tropical forests. Additional protection besides the current approach to conservation of aquatic invertebrates is obviously necessary.

A first step toward conservation of aquatic gastropods is an accurate inventory (Lydeard and Mayden 1995). Efforts toward inventory of aquatic invertebrates in the U.S. have lagged behind inventories of vertebrates although some statewide surveys of aquatic gastropods are appearing (Jokinen 1992, Stewart 2006). Accurate inventory of aquatic gastropods will also encourage studies of the taxonomic, ecological, and general biology of the group (Neves *et al.* 1997). For example, studies of macro-ecological patterns of freshwater gastropods are rare compared with macro-ecological studies of vertebrates. These endeavors have lagged behind other taxa largely because of a lack of descriptive and distributional natural history studies.

Indiana currently lists the conservation status for vertebrate and invertebrate taxa (www.in.gov/dnr/). However, there is currently no specific conservation recognition or protection plan for aquatic gastropods in the state. We recommend such a thorough inventory, recognition, and protection plan for the aquatic gastropods in Indiana. A better understanding of freshwater gastropod ecology demands conservation and further study to protect this valuable natural resource.

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The feeding behavior and diet of an endemic West Virginia land snail, *Triodopsis platysayoides*

Daniel C. Dourson[1]

Belize Foundation for Research and Environmental Education, P.O. Box 129, Punta Gorda, Belize Central America,
bfreemgr@xplornet.com

Abstract: The feeding behavior and diet of the federally threatened land snail *Triodopsis platysayoides* (Brooks, 1933) are reported. The species is atypical among eastern North American land snails in that it remains active and feeding during hot, dry summer months while other land snail species occurring in the region may become motionless or are compelled to estivate. *Triodopsis platysayoides* has also coevolved with a rare mammal, the Alleghany wood-rat, *Neotoma magister*. Clearly, where the wood-rat and *T. platysayoides* coexist, wood-rats furnish a nearly constant food supply to the snail, including wood-rat excrement and a host of wood-rat harvested provisions carried into the snail's location. *Triodopsis platysayoides* includes as part of its diet fungi, lichens, flower blossoms of the tulip tree *Liriodendron tulipifera*, deceased gray cave crickets *Euhadenoecus fragilis*, gray cave cricket excrement, yellow birch *Betula alleghenensis*, and sweet birch *Betula lenta* leaves. Senescent leaves of the birch may form a significant pool of foliar calcium available to the snail in an otherwise acidic environment. *Triodopsis platysayoides* was witnessed feeding on the vacant shells of *Xolotrema denotatum* (Fèrussac, 1821), *Mesomphix cupreus* (Rafinesque, 1831), and its own kind, presumably for the calcium carbonate content, a critical mineral in regulation of bodily functions and shell building. Peak activity for the species occurred after nightfall whereas peak feeding occurred when temperatures were between 18 and 23 °C and relative humidity was between 70% and 85%.

Key words: Cheat threetooth, Cheat River Gorge

The globally rare Cheat threetooth *Triodopsis platysayoides* (Brooks, 1933) is a reclusive snail endemic to the Cheat River Gorge of northern West Virginia, U.S.A. First collected by Graham Netting at Coopers Rock and later described by Stanley Brooks in 1933, the species is associated with interstices of cool boulder talus (Fig. 1), sandstone cliffline features, and to a lesser degree limestone caves within a 21-km stretch of the gorge (Stihler 1994, Hotopp 2006, Dourson 2007). Listed as federally threatened in 1978 by the U.S. Fish and Wildlife Service, the snail is also ranked as a G1 species by NatureServe. A ranking of G1 means that the species is considered Critically Imperiled—at very high risk of extinction due to extreme rarity (NatureServe 2008).

The food preferences of *Triodopsis platysayoides* are poorly known, owing in part to its enigmatic lifestyle, behavior, and feeding preference in the midst of deep rock and boulder talus. Moreover, the preponderance of work to date has largely focused on locating new populations (Hotopp 2000) during daytime hours, a time when the snail is generally less active. Solem (1974) reported that the feeding niche of *T. platysayoides* is apparently among seasonal leaf litter alongside the rocks but made no mention of specific diet. Hotopp and Grimm (1999) cited lichens as a primary food source in the wild. Hotopp (2003) reported that rotting leaf litter appeared to be a food supply in the wild and that lichens may also be consumed, but no unambiguous food taxon was identified. Most reports regarding the diets of land snails are rather vague, largely describing snail diets only in generalities.

In this paper, I report observations on feeding by *Triodopsis platysayoides* that I made during field surveys over 2 years. These observations considerably increase the known foods for this species and, in many cases, provide more specific identification of food organisms than previous reports.

STUDY AREA

The Cheat River Canyon is a steep, 26-km winding stretch along the Cheat River, from Albright in Preston County, northwest to the upper reaches of Cheat Lake in Monongalia County. Elevations of the canyon range from approx. 366 m at the river to approx. 640 m at the uppermost rim of the gorge. The walls of the canyon expose a series of Pennsylvanian and Mississippian sedimentary rock strata deposited 300-350 million years ago. The topmost and thickest layer is composed of Pottsville Sandstone, which outcrops along the rim where tributaries enter the canyon, on periglacial screes and in a variety of other locations. More than halfway down on the slopes of the canyon is the gray or whitish Greenbrier Limestone, in which caves occur.

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[1] Present address: P.O. Box 424, Bakersville, North Carolina 28705, U.S.A.

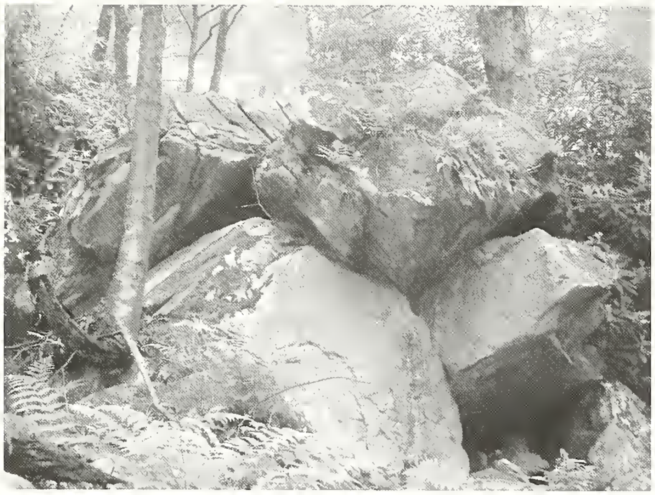

Figure 1. Sandstone boulder and rock talus, considered critical habitat for *Triodopsis platysayoides.*

Oaks, *Quercus,* are ubiquitous, sometimes underlain by dense stands of mountain laurel *Kalmia latifolia.* Oaks dominate upper slopes, while great laurel *Rhododendron maximum,* often found beneath the canopy trees on the shady slopes, typifies wet acidic ravines and the lower slope.

Tulip poplar often dominates the rich cove and lower slope habitats. Along the river, sycamore *Platanus occidentalis* becomes a canopy dominant. The herbaceous vegetation of the canyon is extremely diverse, varied, and depends largely upon the site.

MATERIALS AND METHODS

Visits were conducted at 81 sites where extant populations or the shells of *Triodopsis platysayoides* were documented, providing a range of conditions and possible food sources from which to study feeding behavior. These sites throughout the Cheat River Gorge were studied from May to September of 2006 and 2007, at various times of day and night in a variety of weather conditions. Eighty-nine percent of the sites were visited during daylight hours while eleven percent of the sites sampled were during nighttime hours, which equates to 130 daytime and 17 nighttime visits.

Snails were recorded as active when observed in some form of movement and inactive when the majority of their bodies were retracted inside the shell and the upper and lower tentacles were drawn inward. *Triodopsis platysayoides* was determined to be feeding when its shell and body posture remained somewhat stationary; the lower tentacles were positioned downward and the upper tentacles were shortened and curved backward. On occasion, however, the tentacles would remain mostly extended and in an upright position during feeding behaviors. As *T. platysayoides* fed, irregular head movements were observed and monitored to authenticate actual feeding and not just chemoreception. After feeding was completed, the food was carefully examined for radular abrasions, indicating that the snail had indeed removed and consumed portions of the food.

The foods were reported as senescent, fresh, or in the case of wood-rat guano, both. Food categorized as senescent was noted to be at any visible stage of decomposition. Food retaining living tissue or that did not exhibit signs of decay was classified as fresh. Food sources recognized were identified to genus and, if possible, to species.

Substrate on which the snail fed, time, and weather conditions during the feeding episode were recorded. Temperatures outside rock structure and relative humidity were recorded using a hygrometer. Snails were categorized as either juveniles or adults, by the presence of a reflected lip. Photographs were taken of all feeding behavior and food sources.

Data analysis

Log-ratio χ^2 tests (Christensen 1997) were used to analyze differences in the proportion of observations between day and night surveys. Due to the disproportionate number of daytime visits to nighttime visits, no statistical conclusion was made regarding the snail's diurnal preference for feeding.

RESULTS

Of the 488 live snails documented in 2006 and 2007, 360 were engaged in some form of activity (mostly in movement). The log-ratio χ^2 tests clearly demonstrated that there was an increased activity level seen in *Triodopsis platysayoides* after nightfall when temperatures were 18-24 °C and relative humidity (RH) was above 70%. Twenty-eight percent more snails were active at night than during the day ($\chi^2 = 20.81$, $df = 1$, $P \leq 0.0001$) (Fig. 2). Results from this study also indicate that as temperatures increased and humidity (RH) decreased, *Triodopsis platysayoides* moved deeper into the screes and clifflines. The following accounts are for daytime only. In August (historically the hottest month in West Virginia) of 2006 and 2007, only 7 snails were found within a meter of the surface of the rock structure while 43 snails found in August were found from 2-18 meters deep. July also showed this trend, though to a lesser degree. During the survey months of May, June, and September, the greatest percentage of live snails found (198) occurred within a meter of the surface while (81) of the live snails found were 2-18 meters deep. The month of June found the most live snails (118) within a meter of the surface whereas (25) snails found in June were 2-18 meters deep.

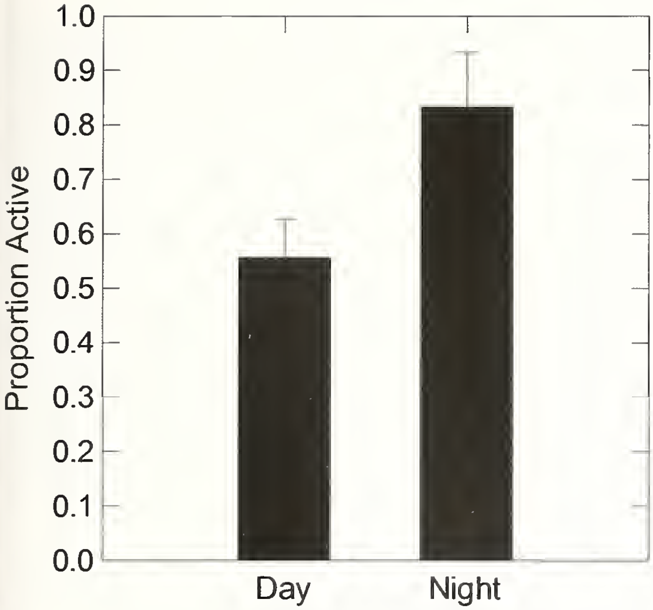

Figure 2. Overall activity levels of *Triodopsis platysayoides* observed in day ($N = 278$) and night ($N = 74$) surveys conducted from May to September 2006 in Monongalia County, West Virginia.

A total of fifty-nine feeding observations were recorded. Twenty-eight of the feeding episodes were documented during nighttime hours from 8:30 pm to 3:30 am, while thirty-one feeding episodes were documented in the daylight hours from 8:00 am to 8:30 pm. The majority of nighttime feeding events (21 out of 28) occurred in temperatures that ranged between 18 and 23 °C (recorded outside the rock structure) and relative humidity (RH) was between 75% and 85% (recorded outside the rock structure). During the daytime 18 out of 31 feeding events occurred between temperatures that ranged between 18 and 23 °C (recorded outside the rock structure), and relative humidity (RH) was between 70% and 85% (recorded outside the rock structure). The highest number of feeding events (35 of 53 or 66%) for both nighttime and daytime hours occurred between 18 and 23 °C with (RH) between 70% and 85%.

The diet of *Triodopsis platysayoides* was a great deal more diverse than reported by Solem (1974) and Hotopp and Grimm (1999). The results of my study documented 27 plant and animal food sources (Table 1) for *T. platysayoides*. The most common meals included senescent birch leaves, gilled mushrooms, and wood-rat scat. Less frequently consumed foods were fresh dead cave crickets, flower petals of great laurel and tulip trees, crustose lichens, and the shells of several species of terrestrial gastropods. Although two species of birch leaves, sweet birch and yellow birch, were the most frequently consumed, red maple and red oak leaves were also documented as food for *T. platysayoides*. The snail diet included senescent portions of at least two fern species, wood fern *Dryopteris intermedia* and hay-scented fern *Dennstaedtia punctiloba*. *Triodopsis platysayoides* also fed on newly emergent fruiting bodies of mushrooms. Wood-rat excrement was eaten fresh (Fig. 3) but also in various stages of decomposition, and the snail also consumed a white mold that grows from wood-rat scats.

Triodopsis platysayoides habitually fed on decaying vegetation. At one location, the species consumed abcissed flower petals of great laurel (Fig. 4) that were in decomposition. The snail appeared to be interested only in the rankest ones. When offered newly fallen flowers, the snail declined to feed on them and instead turned away and headed for the most putrid. At other feeding stations, only the dead or dying portions of fern fronds were eaten. While only three species of Bryophytes (mosses) were documented as food during the study, other species of bryophytes are likely consumed as well, given their frequency in the snails' habitat. Snails fed only on the leafy portions of the moss, intentionally avoiding the more fibrous stems altogether.

Triodopsis platysayoides fed on a variety of mushrooms in the genera *Amanita*, *Tricholoma*, *Russula*, *Hygrophorus*, and *Marasmius*, including several species that had been harvested and transported by wood-rats into the boulder talus habitat. The snail was especially fond of mushroom gills, often crawling upside down to feed on these portions of the fungi. One adult *T. platysayoides* was observed feeding on *Gyroporus castaneus*, a mushroom found fruiting three meters deep in the dark zone of boulder talus. Numerous fungi species that had feeding evidence from snails were observed in dark zones of other rock shelters and talus. Although most were likely fed on by *T. platysayoides*, it is possible that the mushrooms were eaten by other common land snail species such as *Neohelix dentifera* (A. Binney, 1837) or *Triodopsis tridentata* (Say, 1816), sporadically found in talus habitat with *T. platysayoides*.

During the study, the vacant shells of *Mesomphix cupreus* (Rafinesque, 1831) and *Xolotrema denotatum* (Férussac, 1821) were offered to *Triodopsis platysayoides* to determine if the species would utilize the shells as a calcium source. Shells were placed 12 cm in front of two actively crawling *T. platysayoides*. Both snails found the shells within several minutes and promptly fed on the surfaces for more than forty minutes each. After the snails departed, shells on which they fed were examined and showed signs of radula abrasions. On another occasion, an adult *T. platysayoides* was observed feeding (unsolicited) on the vacant shell of its own kind for a period of 30 minutes before it moved on.

The length of feeding episodes appeared to correlate to the solidity of the food source, ranging from 5 minutes to nearly an hour. While grazing on the vacant shell of *Xolo-*

Table 1. Confirmed foods of *Triodopsis platysayoides.*

Scientific name	Common name	Stage of material	# feeding events
Acer rubrum	Red maple leaves	Senescent	3
Amanita flavoconia	a mushroom	Fresh	1
Betula allegheniensis	Yellow birth leaves	Senescent	8
Betula lenta	Sweet birch leaves	Senescent	14
Betula lenta	Sweet birch catkins	Fresh	1
Dennstaedtia punctiloba	Hay-scented fern	Senescent	1
Dryopteris intermedia	Wood fern	Senescent	2
Euhadenacous fragilis	Gray cave cricket	Fresh	2
Euhadenacous fragilis	Grave cave cricket guano	Fresh	1
Gyroporus castaneus	a mushroom	Fresh	1
Hygrophorus sp. (?)	a mushroom	Fresh	1
Liriodendron tulipifera	Tulip tree flowers	Senescent	1
Loeskeobryum brevirostre	a moss	Fresh	1
Marasmius sp. (?)	a mushroom	Fresh	1
Mesomphix cupreus	Copper button shell	Senescent	1
Neotoma magister	Wood rat scat	Both	8
Plagiomnium ciarare	a moss	Fresh	1
Quercus rubrum	Red oak stems	Senescent	1
Quercus rubrum	Red oak leaves	Senescent	1
Rhododendron maximum	Rhododendron flowers	Senescent	1
Russula foetens	a mushroom	Fresh	1
Sassafras albidum	Sassafras leaf	Fresh	1
Thuidium delicatulum	a moss	Fresh	1
Triodopsis platysayoides	Cheat threetooth shell	Senescent	1
Not identified to genus	Crutose lichens	Fresh	1
Not available to genus	Mold on wood rat scat	Fresh	2
Xolotrema denotatum	Velvet wedge shell	Senescent	1

trema denotatum, *Triodopsis platysayoides* took 48 minutes. One juvenile *T. platysayoides* spent 30 minutes feeding on a freshly dead cave cricket. In contrast, *T. platysayoides* spent an average of 15 minutes or less feeding on the tissues of mushroom caps and gills and only 5 minutes on the mold growing from wood-rat scat.

The results of this work found at least a dozen examples of adult and juvenile *Triodopsis platysayoides* feeding on the same foods. For instance, deep within a boulder talus, a fully adult (22 mm) (Fig. 5) and a juvenile (12 mm) were observed feeding together on a recently deceased grey cave cricket. Juvenile and adult *T. platysayoides* were also observed feeding on wood-rat scats, birch leaves, and newly abscised birch catkins. No juveniles of *T. platysayoides* under 8 mm were documented feeding during this study.

DISCUSSION

One of the most important environmental factors to land snails in general is moisture (a frequent cause of death is desiccation). Other factors such as temperature range, escape from predators and disease, and a food source are also important. While most other species of snails fulfill their needs without such a close association with rock, the needs of *Triodopsis platysayoides* are satisfied by certain rock structures in ways that we do not fully understand (Pearce *et al.* 2007). For example, it is not entirely clear where or even if, the species hibernates during winter months. The snail has been recorded 18 meters deep in cave-like caverns that form in sandstone clifflines and boulder talus (Dourson 2007). At this depth, winter temperatures have little effect on the relatively stable microclimate, which generally remains around 14 °C with relative humidity around 70%. During the study, eight snails were observed in May actively feeding in these temperature and (RH) ranges. It is entirely possible that *T. platysayoides* remains active and feeding during winter months, particularly if there are cohabiting woodrats (which do not hibernate) in the same rock features.

Results from the log-ratio χ^2 tests established that there were increased activity levels seen in *Triodopsis platysayoides* after nightfall, suggesting that perhaps peak overall feeding activity occurs at night. However, the 59 feeding events in my study suggest that peak feeding for *T. platysayoides* may be more a function of temperature and relative humidity, not time of day. If so, this is likely a consequence of the snails' specialized habitat that maintains a relatively stable microclimate, allowing the snail to feed during the daytime even if weather conditions outside the rock structure were less than favorable. Both of these hypotheses await further testing.

As a food source, rock and boulder talus habitats of the Cheat River Gorge are comparatively rich, harboring a multiplicity of bryophyte, lichens, trees, a variety of herbaceous vegetation, and a few animal species that are known foods of *Triodopsis platysayoides.* Talus also accumulates large quantities of forest detritus, an ideal medium for growing an assortment of fungi and their fruiting bodies. Moreover, a number of known foods for the species, such as birch leaves as well as tulip tree and rhododendron blossoms, fall easily into the interstitial spaces of the open boulder talus. A snail living in such sites need not travel far for its meal.

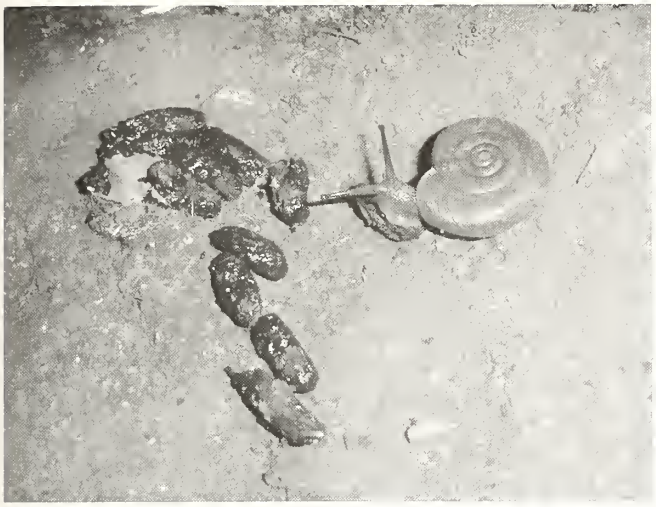

Figure 3. Sub-adult *Triodopsis platysayoides* feeding on fresh Allegheny wood-rat scat. Shell 18 mm in diameter.

Triodopsis platysayoides is a dietary generalist, feeding on a variety of foods largely originating or contained within the screes. Senescent birch leaves were the most habitually consumed by the species (with 22 feeding events). At least ten other senescent materials were included in *T. platysayoides* diet. A number of studies have reported a prevalence of senescent foods in the natural diet of land snails (Richardson 1975, Hatziioannou *et al.* 1994, Iglesias and Castillejo 1999). Many plants contain toxins or refractory compounds such as tannins, so some gastropods eat senescent plant material that has lost many of those secondary compounds (Burch and Pearce 1990). Aging vegetation also concentrates macronutrients in tissues, an example being foliar calcium in birch leaves (Potter *et al.* 1987).

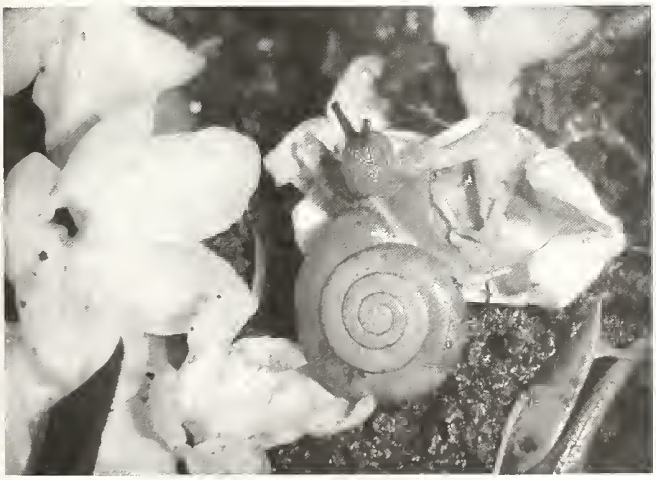

Figure 4. Adult *Triodopsis platysayoides* feeding on aged blossoms of rhododendron. Shell 23 mm in diameter.

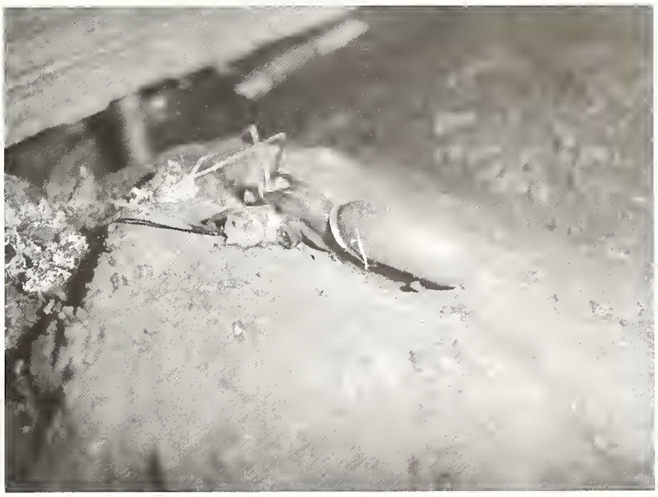

Figure 5. Adult *Triodopsis platysayoides* feeding on recently deceased gray cave cricket. Shell 22 mm in diameter.

Triodopsis platysayoides' most fascinating food relationship is the one it has with the Alleghany wood-rat. In locations where the two species coexist, this affiliation, coupled with the protective sanctuary and microclimate of the talus, has allowed *T. platysayoides* to remain entirely active and feeding inside the talus and cliffs during the hot dry months of summer, particularly in August. As summer temperatures climb, *T. platysayoides* simply moves deeper into talus or cliffline habitats where it remains active and feeding. For other land gastropods during this time, surviving desiccation is the priority. This is because most land snails are not adapted to live in the deep recesses of screes and cliffs. As summer temperatures climb and conditions dry out, surface dwelling snails must retain their vital body moisture by becoming inactive, followed by aestivation.

Clearly, wood-rats benefit *Triodopsis platysayoides* in a number of ways, most importantly as a food supplier. Wood-rats carry into the talus and rock shelters a plethora of known and potential *T. platysayoides* food, including a large assortment of fungi, freshly cut *Dryopteris* species (wood ferns), and scattered, easily accessible deposits of their own excrements. The snail appeared to be especially fond of a white mold growing from aged wood-rat scat (Fig. 6). Wood-rats maintain latrines that are usually sheltered among these rock features providing the snail a near-constant food supply in the relative protection of the screes and cliffs. From a management standpoint, protecting wood-rats and their habitat in the Cheat River Gorge will no doubt benefit the snail.

Hotopp and Grimm (1999) reported that lichen was a

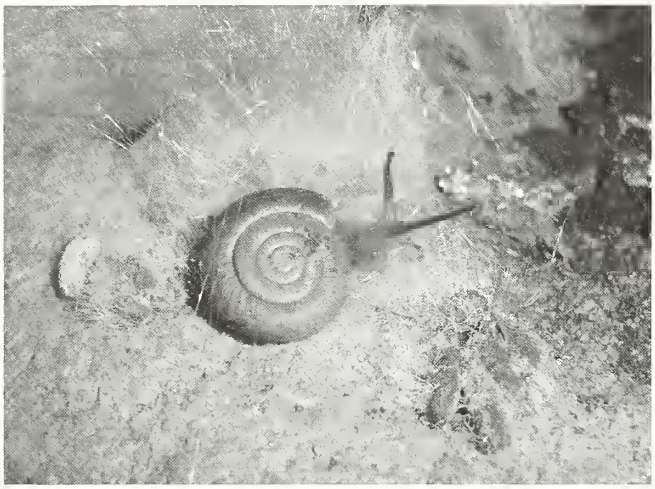

Figure 6. Sub-adult *Triodopsis platysayoides* feeding on mold that grows on wood-rat scat. Shell 18mm in diameter.

primary food source for *Triodopsis platysayoides* in the wild, yet in my study I observed the snail feeding only once on a crustose variety. Lichens are, in fact, eaten by many species of terrestrial snails (Peake and James 1967). The crustose lichen that *T. platysayoides* fed upon was frequent on the surface of outsized sandstone boulders in the Cheat River Gorge and the number of lichen species occurring in the gorge appeared particularly diverse. Because lichen was cited as a primary food source by Hotopp and Grimm in (1999), further investigation into the use of lichens as food by *T. platysayoides* is of special interest.

Diets of juvenile *Triodopsis platysayoides* may differ from those of adults (Caldwell *et al.* 2006). The literature about the different diets in adults and juvenile land snails is largely scarce and ambiguous (Iglesias and Castillejo 1999). Juveniles of *Cepaea nemoralis* (Linnaeus, 1758) are reported to eat some species of plants that the adult animals avoid and *vice versa* (Wolda *et al.* 1971). Other studies have shown no difference in the diets of juveniles and adults of the same species (Wolda *et al.* 1971, Hatziioannou *et al.* 1994, Williamson and Cameron 1996). I found no evidence of such life-stage dietary differences in *T. platysayoides*.

In addition to a food supply, shell-bearing snails require a calcium source for reproduction, regulation of bodily functions, but most importantly shell building. Sandstone boulder and cliffline habitat and their associated soils are reported to be largely calcium deficient environments (Kalisz and Powell 2003). The quest for calcium carbonate sources in these acidic environments are likely more varied than we know, but several suppliers are speculated on here. These include but are not limited to, the vacant shells of gastropods and abscissed leaves of yellow and sweet birch. *Discus macclintocki* (F. C. Baker, 1928), known only from cold rock talus slopes, includes yellow birch leaves in its diet, which may supply a calcium carbonate source for that species. In non-calcareous areas, land snails often appear allied to a calcium supply (Mason 1974). Gosz *et al.* (1973) reported that dead birch leaves could form a significant pool of calcium on the forest floor, since the concentrations of calcium remained high in dead leaves 12 months after abscission. It is not known if the leaves eaten by *T. platysayoides* were consumed for the leaf tissues themselves or perhaps for something growing on the surface of the leaves such as a sooty mold or algae. Leaves were always eaten in their entirety, however, and not just surface-grazed by the snails, suggesting the motive was more for the leaf content.

Besides the 27 documented foods for *Triodopsis platysayoides*, several other foods are suggested here and deserve some mention. Numerous unidentified mushrooms that showed signs of snail feeding, *e.g.*, radula marks and slime trails, were routinely found in *T. platysayoides* habitat and were probably fed on by that species. Fall feeding observations of *T. platysayoides* would likely document additional fungi species, given that fruiting bodies of mushrooms are more frequent during this time of year (Hartman 2003). Rock tripe, a common species of foliose lichen growing on rock surfaces, often exhibited snail-feeding signs close to sites that harbored live *T. platysayoides* and although these lichens were twice offered to the species in the field, the snails declined to feed.

Additional research on the feeding behavior and diet of *Triodopsis platysayoides* will no doubt yield some interesting discoveries. Ideally, studies should be conducted after nightfall, when temperatures are 18-23 °C and relative humidity is 70-85% during late May and early June when the snail is most active and potential food sources are plentiful.

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Structural community changes in freshwater mussel populations of Little Mahoning Creek, Pennsylvania

Eric J. Chapman[1] **and Tamara A. Smith**[2]

[1] Western Pennsylvania Conservancy, Freshwater Conservation Program, Blairsville, Pennsylvania 15717, U.S.A., echapman@paconserve.org

[2] Western Pennsylvania Conservancy, Northwest Field Station, Union City, Pennsylvania 16438, U.S.A., tsmith@paconserve.org

Abstract: As part of a complete biotic survey for Little Mahoning Creek watershed, Western Pennsylvania Conservancy (WPC) used timed searches to survey for freshwater mussels along the entire mainstem of Little Mahoning Creek (56.8 km). The survey captured all historic sites sampled as early as 1905. The 15 sites revealed a high diversity of unionids (10 species) and several rare and endangered taxa, including *Pleurobema sintoxia* (Rafinesque, 1820), *Villosa iris* (I. Lea, 1829), and *Alasmidonta marginata* (Say, 1818). Present survey results indicate an established unionid community with several taxa increasing their distributions compared to historical surveys. Upstream dispersal was halted at the Savan Dam at river kilometer 36.69. No live or dead unionids were found at six sites upstream of the dam. Bray-Curtis similarity indices were calculated between present survey and historical survey accounts. Species composition in the present survey compares favorably (86.14%) with collections by Ortmann (1909) and Bogan and Davis (1992). Several locations have relatively high unionid diversity, despite being located in an area with anthropogenic perturbations.

Key words: Unionids, Bray-Curtis similarity indices, watershed ecology, anthropogenic threats, visual assessment

Little Mahoning Creek, originating near Deckers Point in Indiana County, Pennsylvania, U.S.A., is home to several rare and threatened aquatic species. Most notably, the eastern hellbender salamander *Cryptobranchus alleganiensis* and several rare freshwater mussels and fish are endemic to this watershed. Three of the mussels documented in past surveys of Little Mahoning Creek are presently listed as endangered in Pennsylvania and have global status of either imperiled or vulnerable—*Epioblasma triquetra* (Rafinesque, 1820), *Pleurobema sintoxia* (Rafinesque, 1820), and *Villosa iris* (I. Lea, 1829) (NatureServe 2008). Historic records from the greater Mahoning drainage indicate the presence of the federally endangered *Pleurobema clava* (Lamarck, 1819) and *Obovaria subrotunda* (Rafinesque, 1820) although neither of those species has been found in the Little Mahoning Creek (Ortmann 1919).

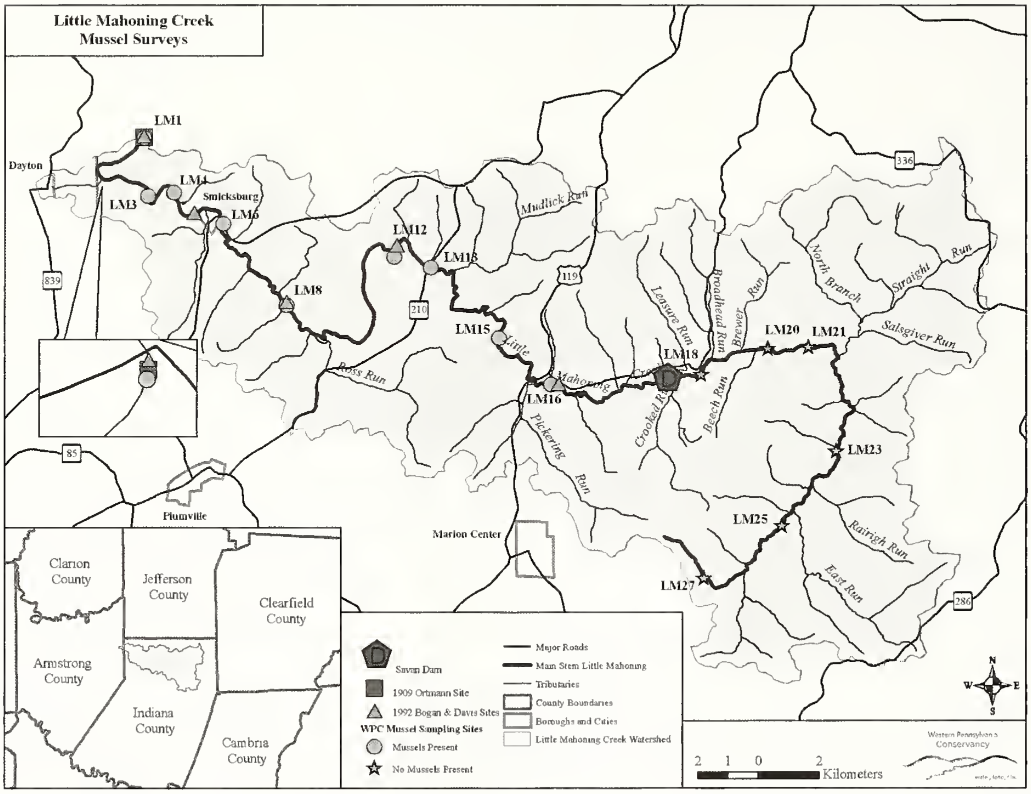
Despite having this diverse unionid fauna, no surveys for freshwater mussels have occurred since 1991, and past surveys did not systematically cover the entire stream length (Ortmann 1909, Bogan and Davis 1992). This study utilized stream-wide surveys to provide information on the distribution and abundance of freshwater mussels along the entire length of Little Mahoning Creek, and to document any changes in species distribution and composition over time. The survey included all historic sites on Little Mahoning Creek that were sampled as early as 1905 (a single site and five in 1991) and filled in gaps by conducting additional surveys throughout the creek.

There are several methods and sampling designs to estimate population sizes of mussels (Strayer and Smith 2003), and a semi-quantitative sampling method was chosen that can detect rare species and give accurate estimates of catch per unit effort (Smith *et al.* 2001). Timed searches indicated mussel species richness in an area as well as catch per unit effort (CPUE). In addition, length data were recorded for each mussel to examine recruitment and age class data. Bray-Curtis similarity indices were calculated to compare results to Bogan and Davis (1992) and Ortmann (1909) to elucidate long-term trends in mussel fauna of this imperiled watershed.

MATERIALS AND METHODS

Study location

Little Mahoning Creek watershed is located in the Pittsburgh Low Plateau Section originating near Deckers Point, Pennsylvania (Fig. 1) and covers an area of 295.61 km^2. Little Mahoning Creek is a tributary to Mahoning Creek, which empties into the Allegheny River near Templeton, Pennsylvania. Agriculture and deciduous forests comprise over 86.9% of the available land area in this watershed. The remaining 13% of the land is used for light industrial and residential uses. The watershed is sparsely populated, with the largest centers being Marion Center Borough (pop. 2,945), Smicksburg Borough (pop. 1,743), and Dayton Borough (pop. 2,302) in 2000 (United States Census Bureau 2007).


Little Mahoning Creek Mussel Surveys
LM1
Dayton
LM3
LM4
Smicksburg
LM6
LM8
LM12
LM13
LM15
LM16
LM18
LM20
LM21
LM23
LM25
LM27
Mudlick Run
Ross Run
Little Mahoning
Pickering Run
Crooked Run
Leasure Run
Broadhead Run
Brewer Run
Beech Run
North Branch
Straight Run
Salsgiver Run
Rairigh Run
East Run
839
210
119
336
85
286
Plumville
Marion Center
Clarion County
Jefferson County
Clearfield County
Armstrong County
Indiana County
Cambria County
Savan Dam
1909 Ortmann Site
1992 Bogan & Davis Sites
WPC Mussel Sampling Sites
Mussels Present
No Mussels Present
Major Roads
Main Stem Little Mahoning
Tributaries
County Boundaries
Boroughs and Cities
Little Mahoning Creek Watershed
N
W
E
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Kilometers
Western Pennsylvania Conservancy

Figure 1. Survey map for the present survey and historical survey locations.

Site selection

To survey the entire mainstem of Little Mahoning Creek and all historic sites methodically, Western Pennsylvania Conservancy (WPC) utilized several Geographic Information System (GIS) applications and searched historical collections. First, a visual assessment was completed, using a modified United States Department of Agriculture (USDA) protocol, which was used to identify substrate and basic stream characteristics that were used as predictors for mussel habitat (*i.e.*, substrate composition, flow, and embeddedness) for the entire mainstem of Little Mahoning Creek, from its confluence with Mahoning Creek upstream to the headwaters in Deckers Point (Fig. 1). Next, all locations that were previously sampled were added to the GIS database. Data from the Bogan and Davis (1992) collections contained latitude and longitude coordinates for all five sites, so it was possible to geo-reference those stations before survey work started in 2007. Museum specimens from the Carnegie Museum of Natural History were searched to identify historical collection sites. Only one collection site was recorded by Ortmann (1909), near the mouth of Little Mahoning Creek at river kilometer (RKM) 0.13 (Fig. 1). Ten additional sites were chosen throughout the creek that had suitable mussel habitat (no bedrock slabs or excessive boulders) and private landowner permission for stream access.

Survey methodology

Freshwater mussel surveys were conducted as in Smith *et al.* (2001). Fifteen sites were surveyed in 2007, using a combination of tactile and visual methods. Although most of the mussels collected were visible at the surface, observers periodically brushed away sediment, flipped over non-embedded rocks, and did some excavation during each search. Surveyors included two people using masks and snorkels and two to five persons surveying with glass bottom buckets. Surveyors collected as many unionid individuals as possible during a specified amount of time, which was dependent upon the width of each site.

Search area was standardized by the effective sampling fraction of 0.06 and a target effective search rate of 0.5 m^2/minute (Smith *et al.* 2001). Sites were standardized to 100 m lengths, and areas were calculated based on an average width at each site. After the area to be surveyed was determined for each 100 m stretch, the following formula was used to determine total search time. Total search time and search area was then divided equally among surveyors, with search times ranging from 35 to 300 minutes.

$$\frac{\text{Search time (min)} = \text{Area (m}^2\text{)} \times \text{(Effective sampling fraction)}}{\text{Effective search rate (m}^2\text{/min)}}$$

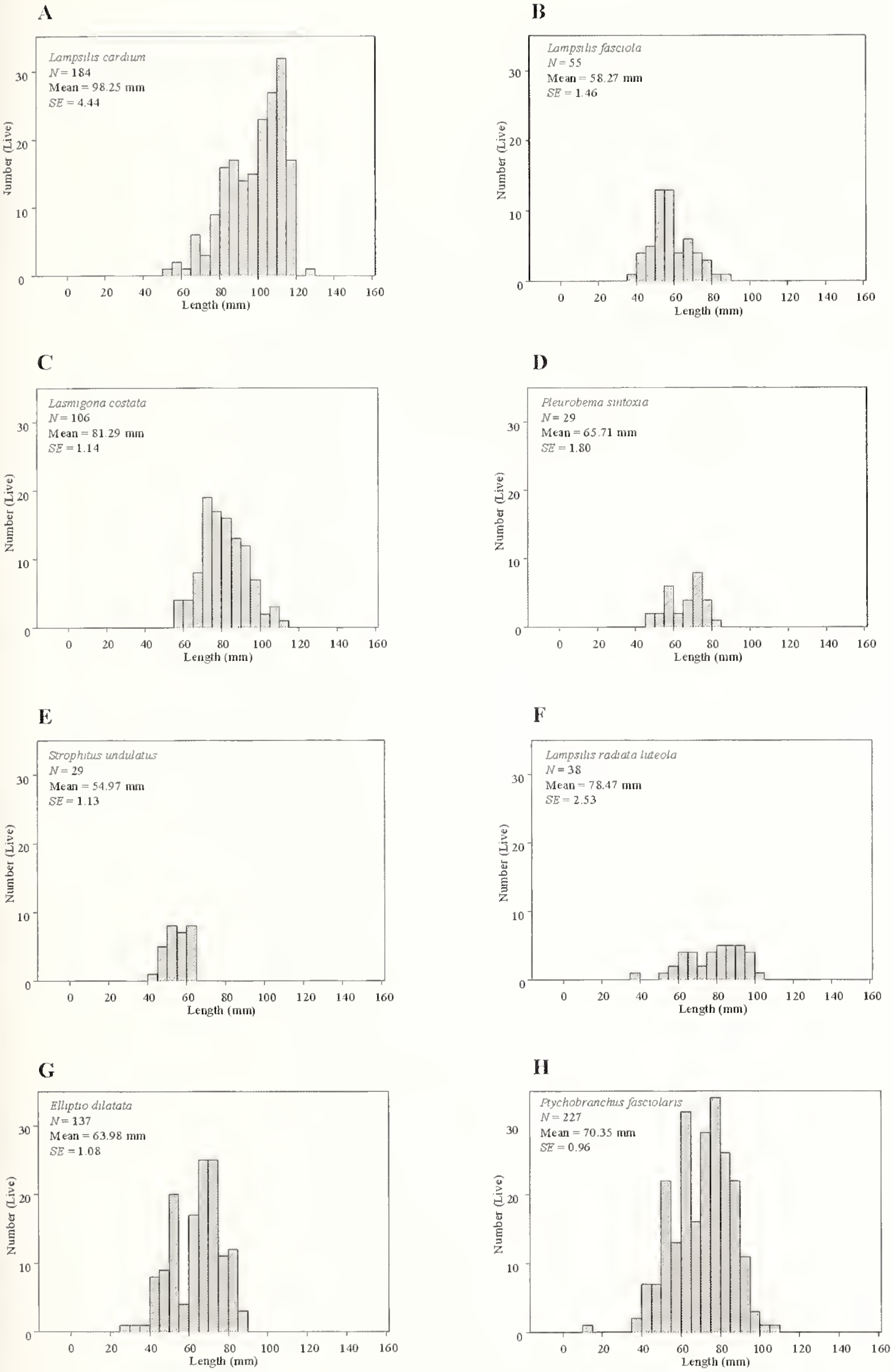

Figure 2. Length-frequency histograms showing size distribution for common species ($N > 10$) found in Little Mahoning Creek.

Sampling started at the downstream end of the study section, and observers moved in an upstream direction covering the entire stream width. Live mussels and dead shells were kept in submersed mesh bags until the survey was finished at each site. Mussels were identified, counted, measured, and released to the study site. Catch per unit effort (CPUE) was calculated as number of unionid individuals collected divided by person-hours (p-h) spent sampling. Shell length of

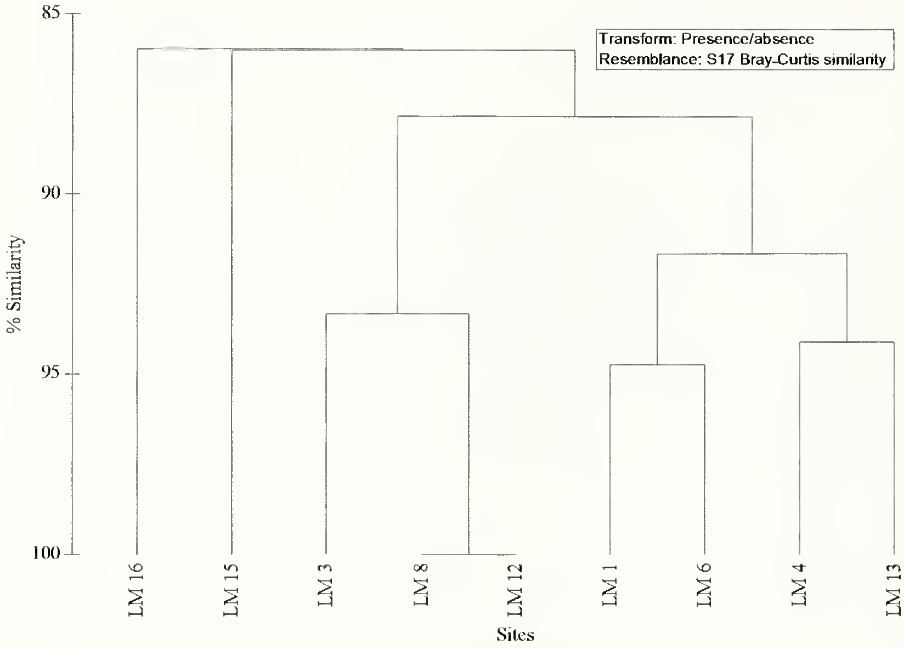

Figure 3. Bray-Curtis similarity dendrogram for the present survey.

each mussel was measured to the nearest 0.1 mm. Length-frequency histograms were created for each species and were examined for obvious breaks indicating year classes. A threshold of 30 mm total length was used to define recent recruitment based on similar studies (Obermeyer 1998, Mohler *et al.* 2006). Mean lengths were calculated for each species at each site to see if there were any longitudinal trends in size distribution. Sex ratios of sexually dimorphic species were also examined.

Statistical analysis

All meristic data collected were analyzed using S-Plus 2000 (1998). Data from all sites were analyzed using PRIMER version 6 (Clarke and Warwick 2001) to determine Margalef's index (d), Pielou's evenness (J'), and Shannon-Wiener diversity index (H') calculated on a log$_2$ scale. All sites were then analyzed for similarity using Bray-Curtis similarity indices. The data were analyzed on two levels. First, data from 2007 were analyzed to see how sites varied within the present survey. Second, data were compared to two historical surveys, Ortmann (1909) and Bogan and Davis (1992). The Bray-Curtis similarity indices determine what surveys and groups of species are most similar as determined by the highest coefficient of similarity (0-100%). Historical survey data were analyzed based on presence-absence counts, which are not skewed by abundant taxa (Clarke and Warwick 2001). Rare taxa thus have the identical weight that dominant species have.

To make comparisons among the three surveys, data from the historical surveys were converted to fit present survey site locations. The 2007 site closest to both historical surveys is shown in parentheses. For example, the author of the survey will appear first, then the date, and finally, the closest 2007 survey point in parenthesis, *i.e.*, Bogan and Davis 1992 (16). Museum specimens from the Carnegie Museum of Natural History were used for the Ortmann collection data, which contained shells from a single sample site LM 1 (RKM 0.13).

RESULTS

Results from this survey indicate an established unionid assemblage in Little Mahoning Creek, with several taxa increasing their distributions in comparison to historical surveys. Using timed searches, we documented 10 species throughout this third order stream.

Total species summary

A total of 812 live mussels and 10 species was found, including several threatened and endangered species (Appendix 1). No additional species were detected as dead shells. All mussels were found at the nine sites located downstream of RKM 31.84; the six sites surveyed between RKM 36.81 and 50.88 were devoid of mussels. Not surprisingly, the least diverse site—LM 16 (RKM 31.84)—was found closest to Savan Dam (Fig. 1). Savan Dam, located at RKM 36.69, appears to be a barrier to upstream dispersal of mussels.

The total number of mussels ranged from 0 to 168 at each site. *Ptychobranchus fasciolaris* (Rafinesque, 1820) was the most abundant species, found at nine sites and accounting for about 28.0% of the total number of mussels found in the timed searches. *Elliptio dilatata* (Rafinesque, 1820), *Lampsilis cardium* Rafinesque, 1820, *Lasmigona costata* (Rafinesque, 1820), and *Lampsilis fasciola* Rafinesque, 1820 were also found at all nine sites that had mussels present (Appendix 1). The aforementioned species accounted for 87.50% of all mussels recovered below Savan Dam. *Pleurobema sintoxia* and *Strophitus undulatus* (Say, 1817) were located at eight of the nine sites, which accounted for an additional 7.2% of identified adults in Little Mahoning Creek. CPUE ranged from 0 to 38.8 mussels/p-h. CPUE was highest at RKM 5.87, with a CPUE of 38.8 mussels/p-h (Ap-

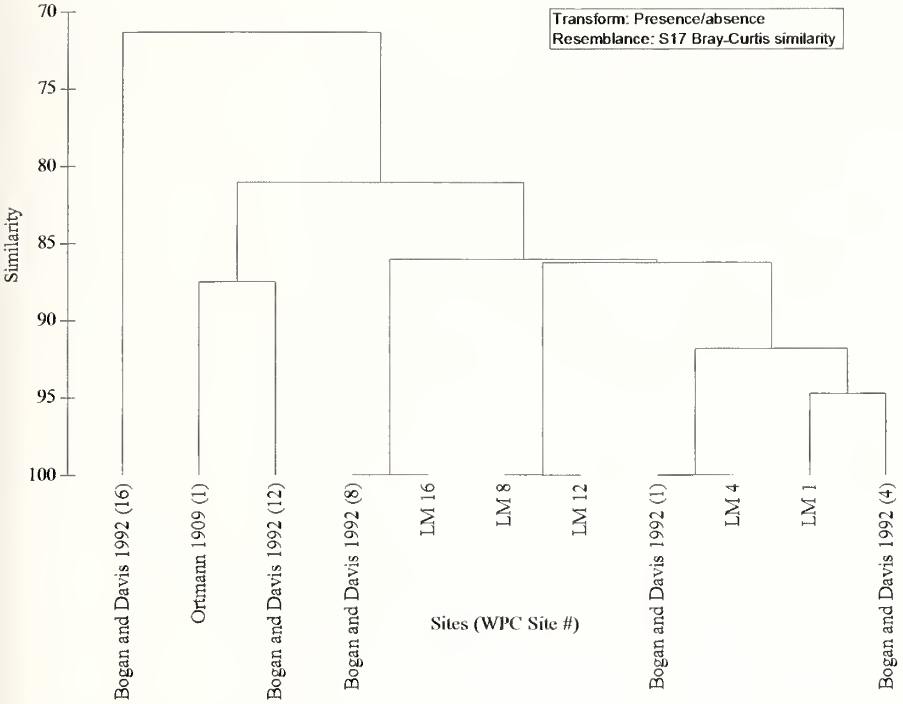

Figure 4. Bray-Curtis similarity dendrogram comparing the present survey with historical surveys.

pendix 1). Except for one 12.5 mm *P. fasciolaris* found at site RKM 20.51 and one 27 mm *Elliptio dilatata* found at RKM 4.38, no evidence of recent recruitment was found (Fig. 2A-H). No significant longitudinal trend in mean shell length was found for adult mussels. Observations of sex ratios in sexually dimorphic species yielded no significant differences among any of the sites surveyed.

Univariate analysis

Margalef's index values (d) varied from a low of 1.34 at LM 8 (RKM 12.57) to a high of 1.91 at LM 3 (RKM 4.38) (Appendix 1). Evenness values were often skewed by a highly successful single taxon, *Ptychobranchus fasciolaris*, which caused lower scores at RKM 20.51 and 31.84. The most diverse site for this survey was RKM 8.38, which had a Shannon-Wiener index of 2.68, while the lowest diversity value was 1.85, found at RKM 31.84.

Similarity indices

Present survey

All sites where mussels were collected (nine sites) during the 2007 field season were compared using the Bray-Curtis similarity indices with presence/absence transformed data (Fig. 2). The sites at LM 8 (RKM 12.57) and LM 12 (RKM 20.51) compared most favorably at 100% similarity. The lowest similarity among all sites was at 85.98% with LM 16 (RKM 31.84) and all other couplets combined. The site at LM 16 (RKM 31.84) was tied with the lowest species count (seven) and had the most uneven community of all sites with d = 0.66 (Appendix 1).

Historical surveys

The overall similarity was high at 71.30% when comparing all 11 sites (Fig. 3). Highest similarity between sites was 100%, which occurred three separate times in this cluster analysis. The highest similarity among the present survey and historical surveys occurs between LM 1 (RKM 0.13) and Bogan and Davis 1992 (4), at 94.74%, which best represents the lower reaches. The single Ortmann survey compares most favorably with Bogan and Davis 1992 (12) at 87.5%, but it still aligns well with all of the lower river sites at 81.06% (Fig. 4).

DISCUSSION

Threats to mussel populations in this watershed include siltation by agriculture and timbering practices (Houp 1993, Brim Box and Mossa 1999), acid mine drainage (PADEP 2007), and hydrologic alterations (Watters 2000). The WPC, in 2006, documented threats to the watershed by utilizing a modified (USDA) rapid bioassessment protocol to assess all 265.49 kilometers of first, second, and third order streams as well as unnamed tributaries in the watershed (Fig. 5). The most significant problem is excessive sedimentation mainly from three sources: farming, natural gas extraction, and dirt and gravel roads (WPC 2007). Farms are contributing sediment to the watershed through improper stream buffer encroachment and cattle directly entering the stream. The excessive sedimentation found at sites LM 12 (RKM 20.51) and LM 16 (RKM 31.84), as identified by the visual assessment and verified by field observation during collections, is effecting mussel diversity, as seen in the depressed evenness values and correspondingly lower Shannon-Wiener scores (Figs. 1, 5, Appendix 1). A second threat is the active natural gas extraction that is rapidly expanding in the watershed. There are currently 2,284 abandoned and/or active gas wells in this small (295.61 km^2) watershed. All wells have access roads that could contribute sediment to the watershed. Third, a

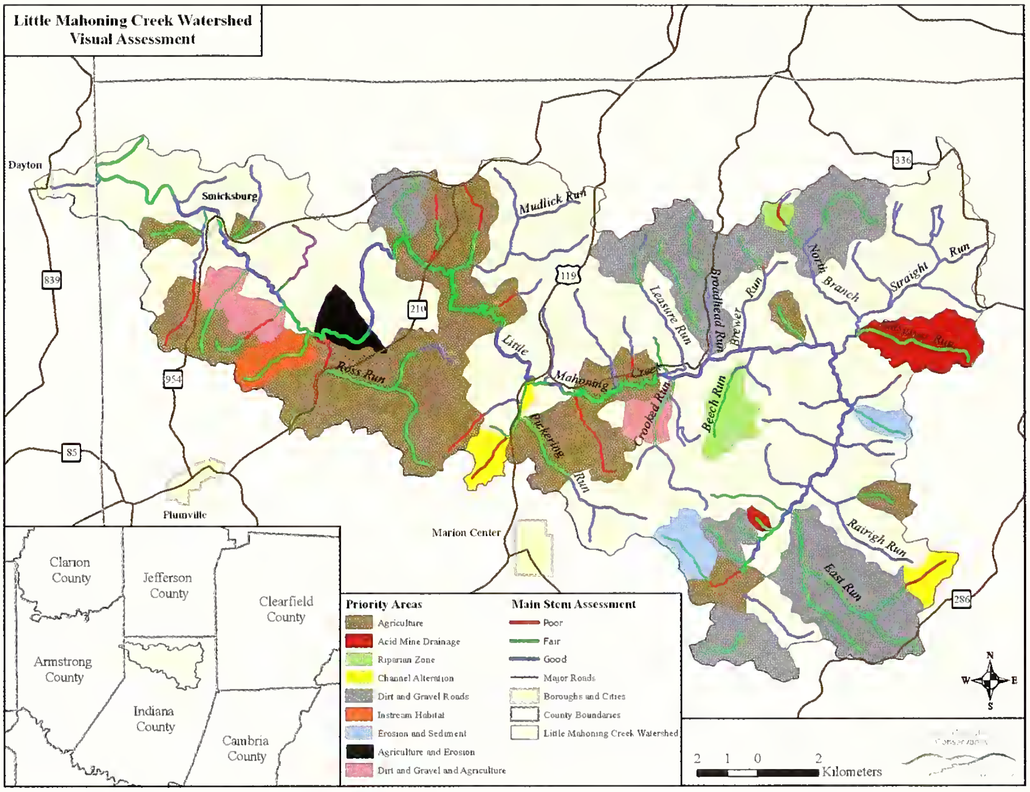
Little Mahoning Creek Watershed
Visual Assessment
Dayton
Smicksburg
Mudlick Run
336
839
119
210
Little
Mahoning
Creek
Ross Run
Leasure Run
Broadhead Run
Brewer Run
North Branch
Straight Run
Beech Run
Crooked Run
Pickering Run
954
85
Plumville
Marion Center
Rairigh Run
East Run
286
Clarion County
Jefferson County
Clearfield County
Armstrong County
Indiana County
Cambria County
Priority Areas
Agriculture
Acid Mine Drainage
Riparian Zone
Channel Alteration
Dirt and Gravel Roads
Instream Habitat
Erosion and Sediment
Agriculture and Erosion
Dirt and Gravel and Agriculture
Main Stem Assessment
Poor
Fair
Good
Major Roads
Boroughs and Cities
County Boundaries
Little Mahoning Creek Watershed
N
W
E
S
2 1 0 2
Kilometers

Figure 5. Visual assessment results detailing problematic areas in Little Mahoning Creek watershed.

large portion, 224.24 km, of the roads are constructed of dirt and gravel, with 32% of these roads causing sedimentation impacts (Fig. 5). WPC is actively working with USDA, Indiana County Conservation District, several township supervisors, and the Pennsylvania Game Commission to repair and mitigate the impacts of these roads to the watershed (ICCD 2008).

Dams prevent dispersal of mussels by preventing upstream movement of their host fish (Watters 1995, 2000). A diverse mussel population was documented in the river downstream of Savan Dam, but no mussels were documented upstream of the dam. Savan Dam has a large head of about two meters, depending on flow, halting upstream migration of host fish. If mussels were present upstream prior to dam construction, it is likely that isolation caused an eventual die-out of the isolated upstream population. Savan Dam was built in 1938 to reduce flooding; however, it is predicted that the stream will eventually meander away from this structure. The WPC is currently negotiating with the landowner to remove the structure to restore unionids to the upper reaches.

Several surveys for unionids completed in this watershed indicate similarity of the historical fauna with that in the present survey. For example, *Elliptio dilatata*, *Lampsilis cardium*, *Lasmigona costata*, *Pleurobema sintoxia*, and *Strophitus undulatus* were collected by all surveys. Species distributions within Little Mahoning Creek also appear to be increasing, because *Lampsilis fasciola* was also found at LM 1 (RKM 0.08) where it was not recorded by Ortmann (1909). In addition to *L. fasciola*, *Lampsilis radiata luteola* was found further upstream than it was reported by Bogan and Davis (1992). These increased ranges could be the result of improvements in water quality (PADEP 2007) or the result of more intensive survey work with additional surveyors in the present survey.

Bogan and Davis (1992) documented the presence of 11 species of unionids in this system, including all the species found in the 2007 study. In addition, two freshly dead and one relict *Epioblasma triquetra* were found by Bogan but not found during 2007 surveys or by Ortmann (1909) (Table 1). Bogan surveyed 11 other sites in the Mahoning Creek drainage in 1991 (five of which are identical to the present survey), but no evidence of *E. triquetra* was recorded at those sites (Bogan and Davis 1992). Given the small numbers, this species has questionable viability in the Mahoning system (Butler 2008). *Obovaria subrotunda* (Rafinesque, 1820) and *Pleurobema clava* (Lamarck, 1819), both historically known from the Mahoning drainage (Ortmann 1919), were not detected in this survey. Most species found in these surveys were relatively large and conspicuous. Two relatively small and cryptically colored species, *Villosa iris* and *Alasmidonta marginata*, were found infrequently by both surveys, with

Table 1. Global and Pennsylvania state ranks for each species found in Little Mahoning Creek. Key to global ranks: G5 = Secure, G4 = Apparently Secure, G3 = Vulnerable, G2 = Imperiled, G1 = Critically Imperiled. Key to state ranks: S5 = Secure, S4 = Apparently Secure, S3 = Vulnerable, S2 = Imperiled, S1 = Critically Imperiled, SNR = not ranked. Ranks according to NatureServe (accessed 4 February 2008).

Species	Global rank	State rank	Ortmann 1909	Bogan 1991	Present survey
Alasmidonta marginata	G4	S4	x	x	x
Elliptio dilatata	G5	S4	x	x	x
*Epioblasma triquetra**	G2 T2	S2		x	
Lampsilis cardium	G5	S4	x	x	x
Lampsilis fasciola	G5	S4		x	x
Lampsilis radiata luteola	G5	S4	x	x	x
Lasmigona costata	G5	S4	x	x	x
Pleurobema sintoxia	G4	S2	x	x	x
Ptychobranchus fasciolaris	G4 G5	S4	x	x	x
Strophitus undulatus	G5	S4 S5	x	x	x
Villosa iris	G5	S1		x	x

* Found by Bogan and Davis (1992) as dead shells only.

the majority only in the lower reaches. The only other small, cryptic mussel was *Strophitus undulatus*, which was found in numerous sites in the present survey, but was absent in the Bogan and Davis survey. The ability to detect small and often overlooked species using timed searches is important and indicates the present survey methods are more rigorous than haphazard collections for finding conspicuous species. In an attempt to classify recent recruitment, excavation is the only possible method to collect an ample numbers of juveniles, and the present survey was not focused on juvenile abundance as much as a complete assessment of unionid diversity in Little Mahoning Creek.

Although substrate was not searched, this survey suggests little recent recruitment. The majority of mussels appear to be older adults, which could however have been a relic from our sampling protocol. Timed searches are less likely to find small individuals than quadrat surveys (Hornbach and Deneka 1996, Vaughn *et al.* 1997). To get a better estimate of recruitment and density and abundance, future studies in the Little Mahoning Creek should use quantitative surveys (Smith *et al.* 2001). Quantitative surveys will also determine if the sex ratios documented were truly representative. Despite the anthropogenic perturbations over the past 100 years, the unionid assemblage remains similar to Ortmann's historical collections. However, the watershed is currently experiencing a level of natural gas exploration that could severely impact this important and intact resource. Future studies should include rigorous quantitative surveys in lower reaches and systematic sampling of excavated quadrates for juveniles, definitely documenting active reproduction or a paucity of juveniles. The WPC is working with local landowners to ensure conservation stewardship within the watershed and to protect this under-appreciated resource in southwestern Pennsylvania.

ACKNOWLEDGMENTS

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Appendix 1. Total number, univariate statistics, number of sites, and catch per unit effort (CPUE, numbers per person-hour) for the present survey.

(Site #) River kilometer	(LM 1) 0.13	(LM 3) 4.38	(LM 4) 5.87	(LM 6) 8.38	(LM 8) 12.57	(LM 12) 20.51	(LM 13) 22.83	(LM 15) 28.17	(LM 16) 31.84	(LM 18) 36.81	(LM 20) 37.86	(LM 21) 40.60	(LM 23) 42.08	(LM 25) 47.39	(LM 27) 50.88	Total numbers	Relative abundance
Species																	
Alasmidonta marginata	1	1		1												3	0.40%
Elliptio dilatata	7	5	16	21	15	11	36	12	14							137	16.90%
Lampsilis cardium	21	8	66	24	23	15	16	6	5							184	22.70%
Lampsilis fasciola	3	3	12	11	8	4	8	3	3							55	6.80%
Lampsilis radiata luteola	19		5	5			5	1	3							38	4.70%
Lampsilis costata	51	2	32	1	4	1	10	4	1							106	13.10%
Pleurobema sintoxia	1	1	8	6	5	1	5		2							29	3.60%
Ptychobranchus fasciolaris	18	17	21	36	32	19	34	11	39							227	28.00%
Strophitus undulatus	6	2	6	8	2	1	2	2								29	3.60%
Villosa iris			2	2												4	0.50%
Total numbers (*N*)	127	39	168	115	89	52	116	39	67	0	0	0	0	0	0	812	
S	9	8	9	10	7	7	8	7	7	0	0	0	0	0	0		
d	1.65	1.91	1.56	1.90	1.34	1.52	1.47	1.64	1.43	0	0	0	0	0	0		
J'	0.77	0.79	0.81	0.81	0.83	0.76	0.83	0.87	0.66	0	0	0	0	0	0		
H' ($\log_2$)	2.44	2.37	2.5	2.68	2.34	2.14	2.50	2.43	1.85	0	0	0	0	0	0		
Search time (min)	260	260	260	300	300	300	240	260	200	200	132	100	100	35	35		
CPUE (#/p-h)	29.3	9	38.8	23	17.8	10.4	29	9	20.1	0	0	0	0	0	0		
Area (m^2)	2286	2439	3170	1890	2713	2134	1951	2134	1219	1280	1219	1524	762	487	335		

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Diversity and distribution of freshwater gastropods in the Bayou Bartholomew drainage, Arkansas, U.S.A.

Russell L. Minton[1], John D. White[1], David M. Hayes[1,*], M. Sean Chenoweth[2], and Anna M. Hill[1]

[1] Department of Biology, University of Louisiana at Monroe, 700 University Avenue,
Monroe, Louisiana 71209-0520, U.S.A., minton@ulm.edu

[2] Department of Geosciences, University of Louisiana at Monroe, 700 University Avenue, Monroe, Louisiana 71209-0550, U.S.A.

Abstract: Bayou Bartholomew is a low-gradient river system that drains much of southeastern Arkansas and northeastern Louisiana, U.S.A. As one of the few southeastern streams remaining un-impounded, the Arkansas reach of the bayou harbors a rich freshwater molluscan fauna. Collecting efforts have historically focused on documenting freshwater mussel and fish diversity, and there was no prior survey focusing on freshwater gastropods. This survey of the drainage yielded 13 gastropod species representing three orders and seven genera. Pulmonates were most abundant in low-order reaches of the drainage, while gill-breathing snails dominated higher-order reaches. Co-occurrence analyses indicated that pulmonates occurred significantly more often with other pulmonates than they did with gill-breathers; this trend was also observed in gill-breathers. Both stream order and predominant substrate influenced species richness and abundance. Our findings were consistent with other published studies on freshwater snail distribution but may be confounded by drought conditions experienced during the survey.

Key words: snails, ecology, drought, co-occurrence

The southern United States harbors one of the most diverse freshwater mollusc assemblages in the world (Williams *et al.* 1993, Neves *et al.* 1997). Of the over 800 species of freshwater gastropods in North America (Lysne *et al.* 2008), up to 40 are reported from Arkansas (Hayes 2008, NatureServe 2008), depending on source. Like many freshwater taxa, snails are experiencing declines due to habitat degradation, pollution, and anthropogenic effects (Lydeard *et al.* 2004, Perez and Minton 2008). Few stream systems in the United States with abundant snail resources have remained unaltered or unexploited. Those rare, minimally impacted systems offer a glimpse into the natural condition that existed prior to widespread impoundment, channelization, and other human influences.

Bayou Bartholomew originates in loess hills west of Pine Bluff, Arkansas and flows 457 km through Jefferson, Lincoln, Drew, Desha, and Ashley counties in Arkansas and Morehouse Parish in Louisiana before its confluence with the Ouachita River near Sterlington, Louisiana. It is the only non-channelized river in southeast Arkansas and northeast Louisiana. Bayou Bartholomew is a low-gradient, Yazoo-type watershed occupying approx. 20% of the Ouachita River basin and draining over 400,000 ha in southeast Arkansas and northeast Louisiana (Broom 1973), including parts of the Arkansas River floodplain (Saucier 1994). Upstream reaches of the drainage are subject to frequent drying and modification due to low water levels; single reaches can become unconnected chains of pools during summer months. Most of Bayou Bartholomew occurs within the Mississippi Alluvial Basin ecoregion characterized by fine textured and fertile alluvial soils well suited to agricultural development (Alley 2005). Agricultural fields, row crops, and pastures dominate land-use in Bayou Bartholomew's watershed. There is a narrow riparian zone along the river, in most cases less than 50 m wide, dominated by bottomland hardwood species such as water tupelo (*Nyssa aquatica*), bald cypress (*Taxodium distichum*), and maples (*Acer* spp.). Erosion, sedimentation, agriculture and urban nutrient inputs, and irrigation water withdrawals have been the main stressors of the Bayou Bartholomew stream ecosystem for many years (Alley 2005).

Most research conducted on Bayou Bartholomew has focused on fishes (Thomas 1976, Hutchins 1988, Pezold *et al.* 2002) and mussels (George and Vidrine 1993, Pezold *et al.* 2002, Brooks *et al.* 2005). These surveys found that Bayou Bartholomew harbors a diverse mussel assemblage in both Arkansas and Louisiana. However, exact figures for the number of gastropod taxa in Bayou Bartholomew are lacking. The objectives of this project were (1) to assess the current status, diversity, and distribution of snail species in the Arkansas portion of Bayou Bartholomew and (2) to provide baseline data for monitoring these species in the future. We additionally explored potential population structure in

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[*] Current Address: Department of Biological Sciences, Arkansas State University, P.O. Box 599, State University, Arkansas 72467-0599, U.S.A.

the drainage and assessed any roles that substrate and stream order had on diversity and distribution.

MATERIALS AND METHODS

The Arkansas portion of the Bayou Bartholomew drainage was surveyed from August 2004 to April 2006. Seventy-four sites were surveyed between the headwaters west of Pine Bluff, Arkansas and the Arkansas-Louisiana state line (Fig. 1, Appendix 1). At each site, the predominant substrate encountered was classified as mud, silt, sand, clay, gravel, or rock; dry sites were recorded separately. One person conducted an hour-long visual search at each site, and all live snails encountered were collected, identified to species, and returned to the river; dead shells were not included in the survey. Voucher specimens of all species were preserved in 95% ethanol and are housed at the ULM Museum of Natural History and await cataloguing. Nomenclature and classification followed Turgeon *et al.* (1998). The status of each species was determined by comparing our findings to global heritage ranks given on NatureServe (2008) and state heritage ranks provided by the Arkansas Heritage Program (2008).

Because the survey was performed during a period of extreme drought (National Weather Service 2008), only sites where live snails were present were used for statistical analyses. We felt this was appropriate because we had no way of establishing if sites without snails indicated a true lack of gastropods, or if snails had moved out of those areas or died.

The influence of environmental factors on snail diversity and distribution was assessed using one-way analyses of variances to determine the effects of stream order and predominant substrate on the numbers of pulmonates and caenogastropods and total richness at each site in JMP 7.0 (SAS Institute, Cary, North Carolina). To test for potential structuring within the watershed, co-occurrence analyses using species abundances at each site were performed in EcoSim 7 (Gotelli and Entsminger 2004) using the C-score, checkerboard species pairs, and number of species combinations settings with fixed row and column sums and all other settings as defaults. C-score calculates co-occurrence values according to a model based on Stone and Roberts (1990) and compares observed occurrences with a set of simulated datasets; if the observed value is significantly larger than the random simulations, significant co-occurrence exists. Checkerboard pair analyses calculate how many species never occur together in the observed and simulated data; if

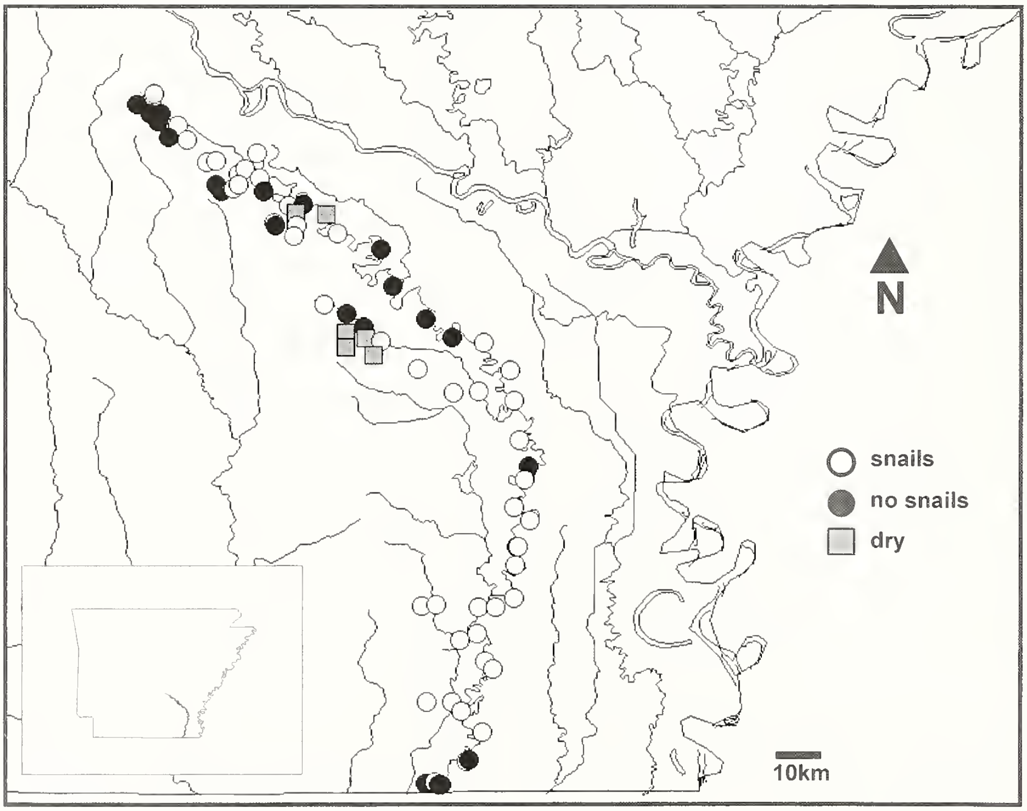

Figure 1. Map of survey sites in the Bayou Bartholomew drainage of Arkansas. Filled circles indicate sites where snails were present, open circles where snails were absent, and gray squares indicate sites that were dry. Inset map shows the location of the drainage in the state.

the observed value is larger, then singleton species that do not occur with any others may be biasing the analysis toward significant structuring (Diamond 1975). Number of species combinations analyses calculate the number of unique species that co-occur according to Pielou and Pielou (1968). If the observed number of species combinations is greater than random, species co-occur at a significant level.

RESULTS

Of the 74 sites surveyed, 8% were dry, 28% had water but no snails, and 64% of the sites had snails (Fig. 1). A total of 3,384 individual snails representing 13 species, 12 genera, seven families, and three orders were found throughout the drainage (Table 1). These collections represent 35% of the previously reported state fauna; no new state records or alien species were found. *Pleurocera canaliculata* (Say, 1821) was the most abundant species, with 2,230 individuals (66% of total) surveyed. Snails were most abundant at site 72 in Ashley County, with 630 individuals representing three species. The highest species richness in the drainage was at site 57, Bearhouse Creek in Ashley County, with six species. The highest species richness for the main stem of Bayou Bartholomew was at sites 7 and 65 in Jefferson and Ashley Counties, each with five species. All species encountered have G4-G5 global heritage rankings, indicating overall stability; however, these same species hold SU state rankings, meaning their status in Arkansas is unknown.

Neither stream order (1-way ANOVA, $F = 1.54$, $df = 45$, $P > 0.21$) nor predominant substrate ($F = 0.855$, $P > 0.52$) had significant effects on total species richness. Stream order, however, had significant effects on the numbers of pulmonates ($F = 3.45$, $P < 0.03$) and caenogastropods ($F = 4.19$, $P < 0.02$); fourth-order reaches had more caenogastropods and fewer pulmonates than first through third-order reaches. Predominant substrate showed no effect on the number of pulmonates ($F = 0.72$, $P > 0.61$) but had a significant effect on the number of caenogastropods ($F = 4.78$, $P < 0.002$). Caenogastropods were significantly more abundant in areas with sandy substrate than in any other. Co-occurrence analyses based on C-scores indicated that there was a significant species structure in the watershed, with pulmonates occurring with other pulmonates more frequently than with caenogastropods and *vice versa* ($P < 0.001$). Analysis of species combinations additionally suggested significant structuring ($P < 0.02$), but analysis of checkerboard species pairs also indicated that singleton species presence was biasing the data toward co-occurrence ($P < 0.04$).

Table 1. Gastropod taxa found in the Bayou Bartholomew drainage of Arkansas. Nomenclature and classification follow Turgeon *et al.* (1998).

ARCHITAENIOGLOSSA (gill-breathing)		
	Viviparidae	*Campeloma decisum* (Say, 1817)
		Viviparus intertextus (Say, 1829)
		Viviparus subpurpureus (Say, 1829)
BASOMMATOPHORA (pulmonate)		
	Ancylidae	*Ferrissia rivularis* (Say, 1817)
		Laevapex fuscus (Adams, 1841)
	Lymnaeidae	*Fossaria bulimoides* (Lea, 1841)
		Pseudosuccinea columella (Say, 1817)
	Physidae	*Physella gyrina* (Say, 1821)
	Planorbidae	*Helisoma anceps* (Menke, 1830)
		Micromenetus dilatatus (Gould, 1841)
		Planorbella trivolvis (Say, 1817)
NEOTAENIOGLOSSA (gill-breathing)		
	Hydrobiidae	*Cincinnatia integra* (Say, 1829)
	Pleuroceridae	*Pleurocera canaliculata* (Say, 1821)

DISCUSSION

The diversity of snails in low gradient, Mississippi alluvial and gulf coast rivers remains relatively unstudied. The few published studies addressing this point (*e.g.*, Gordon 1985, Gordon *et al.* 1993-1994) indicate these aquatic systems possess low snail diversity (up to 15 species; Gordon *et al.* 1993-1994) due to many factors, including a prevalence of soft substrate reaches, low flow, and frequent seasonal drying. The species richness we observed in Bayou Bartholomew is consistent with these values, and we speculate the observed distribution reflects variation in the temporal water levels in the river. The co-occurrence analyses indicated that pulmonates were found with other pulmonates at a significantly higher rate than with caenogastropods and *vice versa.* Analyses of variance showed these groups were associated with stream order, with pulmonates occupying lower order reaches of the bayou and caenogastropods occupying higher order reaches.

Bayou Bartholomew is a highly variable system with periodic drying which would tend to decrease overall species richness. All of the species found in Bayou Bartholomew have broad distributions (Burch and Tottenham 1980, NatureServe 2008) and almost all are from groups of taxa known to be highly resistant to desiccation and drought (Pilsbry 1896, Alyakrinskaya 2004) and indicative of temporary environments (Eckblad 1973). These same taxa frequently characterize lentic and other low flow areas (Brown *et al.* 1998). Therefore, the bayou system may be well suited to species adapted to the high disturbance conditions that have characterized Bayou Bartholomew for the last 5,000-7,000 years (Saucier 1994).

Other longer-term factors may also be influencing snail diversity in Bayou Bartholomew. The watershed is a relatively young system and, as such, would generally possess a less diverse fauna than older systems such as the large rivers of the North American interior (Saucier 1994). The upper reaches of the bayou were originally connected to the Arkansas River and began to separate into a different drainage approximately 15,000 years ago following the last glacial maxima. Subsequent flooding and sediment deposition caused the Arkansas River to divert into its current channel, and the abandoned channel connected to the lower portion of the bayou 5,000 to 7,000 years ago.

The National Weather Service in Little Rock, Arkansas reported that an extreme drought began in 2005 and ended late in 2006. By the end of 2005, average precipitation deficits for the state were 35.5 cm below normal; some areas in the drainage near the Arkansas and Louisiana state line were over 50 cm below normal (National Weather Service 2008). These conditions caused many of the low-order tributaries in the drainage to dry out completely, while many higher order reaches became isolated pools. Brown *et al.* (1998) hypothesized that pulmonate species were more tolerant to temporary streams and extreme conditions, and would therefore dominate such areas. We think it is likely that distribution of snails we observed was strongly influenced by intense drought conditions during our survey. Our data indicate a similar general pattern in Bayou Bartholomew, with pulmonate species being more prevalent upstream and in tributaries, and gill-breathing species occupying higher order downstream areas that are presumably deeper and more stable. Pulmonates also exhibit greater dispersal abilities and can re-colonize dry areas more easily (Davis 1982, Brown 1991).

While many natural factors played a role in the observed diversity and abundance of snails in the bayou, these may have been confounded by our survey methods. A single man hour was dedicated to surveying at each site, and this may have decreased the probability of finding small species like hydrobiids and freshwater limpets. Most limpets were found associated with discarded glass beer bottles; visual inspection found many in bottlenecks. Given their small size and abundance of human-produced refuse at many points in the drainage, species like limpets could be easily overlooked. Additionally, some species including viviparids bury themselves in soft substrates (Van Cleave and Altringer 1937) and individuals may have been missed in the survey. Subsequent surveys during periods of higher water employing more effort may well provide more accurate estimates of richness and distribution; however, despite the drought and limited available effort, we feel our results are consistent with published data.

Modern assessments of freshwater mollusc populations, particularly snail populations serve two important purposes. First, distribution and status surveys provide baseline data for tracking population fluctuations and declines (Hartfield and Rummel 1985, Blalock and Sickel 1996, Lydeard *et al.* 1999, Vaughn and Taylor 1999). Second, these studies can reveal biotic and abiotic interactions that may be influencing snail community structure (Brown *et al.* 1998) for further experimental examination. Since many freshwater molluscs are experiencing their highest declines in terms of richness and abundance (Neves *et al.* 1997), it is critical that surveys such as ours are performed to serve as baseline data documenting biological diversity.

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Appendix 1. Survey localities in the Bayou Bartholomew drainage. Site numbers follow Fig. 1. Sites that were dry are indicated as such.

Site	Locality
1	Bayou Bartholomew at Hardin Road, Jefferson County 34°15′33″N, 92°09′02″W
2	Unnamed tributary at Gorman Road, Jefferson County 34°16′21″N, 92°08′39″W

Site	Locality
3	Unnamed tributary, Jefferson County 34°15′15″N, 92°10′30″W
4	Bayou Bartholomew at Princeton Pike Road, Jefferson County 34°14′09″N, 92°08′00″W
5	Unnamed tributary at Hardin-Reed Road, Jefferson County 34°14′12″N, 92°09′04″W
6	Unnamed tributary at Brantley Road, Jefferson County 34°13′20″N, 92°08′19″W
7	Bayou Bartholomew, Jefferson County 34°12′51″N, 92°06′11″W
8	Nevins Creek at Beechnut Road, Jefferson County 34°11′38″N, 92°07′08″W
9	Nevins Creek at SR 54, Jefferson County 34°11′19″N, 92°05′03″W
10	Boggy Bayou at Brinkley Road, Jefferson County 34°08′55″N, 92°03′02″W
11	Boggy Bayou at Middle Warren Road, Jefferson County 34°09′07″N, 92°02′01″W
12	Boggy Bayou at SR 15, Jefferson County 34°07′42″N, 91°59′30″W
13	Bayou Bartholomew at Bohannon Road, Jefferson County 34°08′14″N, 91°58′52″W
14	Bayou Bartholomew at Ohio Street, Jefferson County 34°09′56″N, 91°57′37″W
15	Sandy Bayou at Middle Warren Road, Jefferson County 34°06′30″N, 92°01′55″W
16	Unnamed tributary at Ty Lane, Jefferson County 34°05′46″N, 92°01′25″W
17	Sandy Bayou at SR 54, Jefferson County 34°06′02″N, 92°00′05″W
18	Sandy Bayou at SR 15, Jefferson County 34°06′34″N, 91°59′36″W
19	Bayou Bartholomew at Gibb-Anderson Road, Jefferson County 34°07′16″N, 91°57′31″W
20	Bayou Bartholomew at Gibb-Anderson Road, Jefferson County 34°05′45″N, 91°56′51″W
21	Bayou Bartholomew at CR 70, Lincoln County 34°04′19″N, 91°54′03″W
22	Bayou Bartholomew at CR 70, Lincoln County 34°04′25″N, 91°52′38″W
23	Melton Creek at CR 10, Lincoln County 34°02′24″N, 91°55′46″W
24	Flat Creek at CR 10, Lincoln County 34°02′06″N, 91°55′36″W
25	Flat Creek at CR 71, Lincoln County 34°03′21″N, 91°53′18″W
26	Flat Creek at CR 11, Lincoln County 34°03′18″N, 91°50′01″W (DRY)
27	Unnamed tributary at CR 8, Lincoln County 34°01′48″N, 91°53′25″W (DRY)
28	Turtle Creek at CR 8, Lincoln County 34°00′34″N, 91°53′32″W
29	Bayou Bartholomew at US 425, Lincoln County 34°01′12″N, 91°48′53″W
30	Bayou Bartholomew at CR 2, Lincoln County 33°59′33″N, 91°44′14″W
31	Bayou Bartholomew at SR 293, Lincoln County 33°55′33″N, 91°42′58″W
32	Ables Creek at SR 54, Lincoln County 33°53′33″N, 91°50′15″W
33	Ables Creek at CR 32, Lincoln County 33°52′33″N, 91°47′54″W
34	Ables Creek at Gentry Road, Lincoln County 33°51′08″N, 91°46′03″W
35	Bayou Bartholomew at SR 54, Lincoln County 33°51′59″N, 91°39′23″W
36	Chance Creek at CR 32, Lincoln County 33°50′22″N, 91°47′52″W (DRY)
37	Chance Creek at SR 54, Lincoln County 33°49′48″N, 91°45′44″W (DRY)
38	Lyle Creek at CR 32, Lincoln County 33°48′48″N, 91°47′48″W (DRY)
39	Chance Creek at SR 83, Lincoln County 33°49′30″N, 91°44′07″W
40	Bayou Bartholomew at SR 293, Lincoln County 33°50′00″N, 91°36′31″W
41	Bayou Bartholomew at CR 20, Desha County 33°49′27″N, 91°33′07″W
42	Weaver Creek at SR 83, Lincoln County 33°48′03″N, 91°44′51″W (DRY)
43	Prairie Creek at Prairie Creek Road, Drew County 33°46′35″N, 91°40′09″W
44	Bayou Bartholomew at SR 138, Drew County 33°46′24″N, 91°30′17″W
45	Panther Creek at SR 293, Drew County 33°43′56″N, 91°36′16″W
46	Ables Creek at SR 138, Drew County 33°44′10″N, 91°33′41″W
47	Bayou Bartholomew at CR 77, Drew County 33°43′11″N, 91°29′47″W
48	Bayou Bartholomew, Drew County 33°38′52″N, 91°29′09″W
49	Bayou Bartholomew at Fourmile Creek Road, Drew County 33°36′03″N, 91°28′14″W
50	Bayou Bartholomew, Drew County 33°34′32″N, 91°28′41″W
51	Bayou Bartholomew at SR 35, Drew County 33°31′40″ N 91°29′51″W
52	Bayou Bartholomew at Hill Community Road, Drew County 33°30′10″N, 91°28′05″W
53	Bayou Bartholomew, Drew County 33°27′17″N, 91°29′30″W
54	Bayou Bartholomew at Panther Break Road, Drew County 33°27′16″N, 91°29′23″W
55	Bayou Bartholomew, Drew County 33°25′22″N, 91°29′41″W
56	Jordan Creek at SR 61, Ashley County 33°20′52″N, 91°39′49″W

Site	Locality
57	Bearhouse Creek at SR 61, Ashley County 33°21′02″N, 91°38′08″W
58	Little Bayou at CR 104, Ashley County 33°20′50″N, 91°33′39″W
59	Bayou Bartholomew at CR 104, Ashley County 33°20′49″N, 91°31′50″W
60	Bayou Bartholomew, Ashley County 33°21′44″N, 91°29′51″W
61	Overflow Creek at US 82, Ashley County 33°17′10″N, 91°35′36″W
62	Bayou Bartholomew at US 82, Ashley County 33°17′54″N, 91°33′44″W
63	Bayou Bartholomew at Mount Pleasant Road, Ashley County 33°14′55″N, 91°32′55″W
64	Bayou Bartholomew below SR 278, Ashley County 33°14′09″N, 91°32′06″W
65	Beach Creek at SR 8, Ashley County 33°10′28″N, 91°39′14″W
66	Overflow Creek at SR 8, Ashley County 33°10′27″N, 91°36′31″W
67	Bayou Bartholomew, Ashley County 33°09′30″N, 91°35′27″W
68	Bayou Bartholomew at SR 8, Ashley County 33°07′16″N, 91°33′13″W
69	Bayou Bartholomew, Ashley County 33°04′03″N, 91°34′51″W
70	Bayou Bartholomew at SR 173, Ashley County 33°04′16″N, 91°34′41″W
71	Bayou Bartholomew at CR 365, Ashley County 33°01′37″N, 91°39′22″W
72	Bayou Bartholomew, Ashley County 33°01′47″N, 91°38′19″W
73	Bayou Bartholomew, Ashley County 33°01′30″N, 91°37′36″W
74	Bayou Bartholomew, Ashley County 33°01′31″N, 91°37′52″W

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RESEARCH NOTE

Conus lightbourni holotype returned to the Delaware Museum of Natural History

Elizabeth K. Shea[1] **and William J. Fenzan**[2]

[1]Mollusk Department, Delaware Museum of Natural History, 4840 Kennett Pike, Wilmington, Delaware 19807, U.S.A., eshea@delmnh.org

[2]401 Sinclair St., Norfolk, Virginia 23505-4359, U.S.A., bill@fenzan.com

Key words: Bermuda, Conidae

In the early 1970s, the deep water gastropod fauna of Bermuda was sampled using baited lobster traps (Lightbourn 1991). This novel approach yielded many unusual gastropods, many of which were provided to Dr. R. Tucker Abbott at the Delaware Museum of Natural History (DMNH) for further study and identification. Several new species were described based on these specimens, including *Conus (Floraconus) lightbourni* Petuch, 1986. The DMNH was identified as the type repository for *C. lightbourni* and catalog numbers were assigned for the holotype (DMNH 134938) and paratype (DMNH 134939) lots; however, the holotype was never received (Bieler and Bradford 1991). There was considerable speculation regarding the location of this specimen for the past 20 years. No neotype was ever designated.

The original description of *Conus lightbourni* was based on 9 specimens examined, all collected south of Castle Island, Bermuda by Jack Lightbourn and Arthur Guest. Petuch (1986) described the holotype as 35 mm long and 16 mm wide, having a bright orange base color, salmon-pink spire,

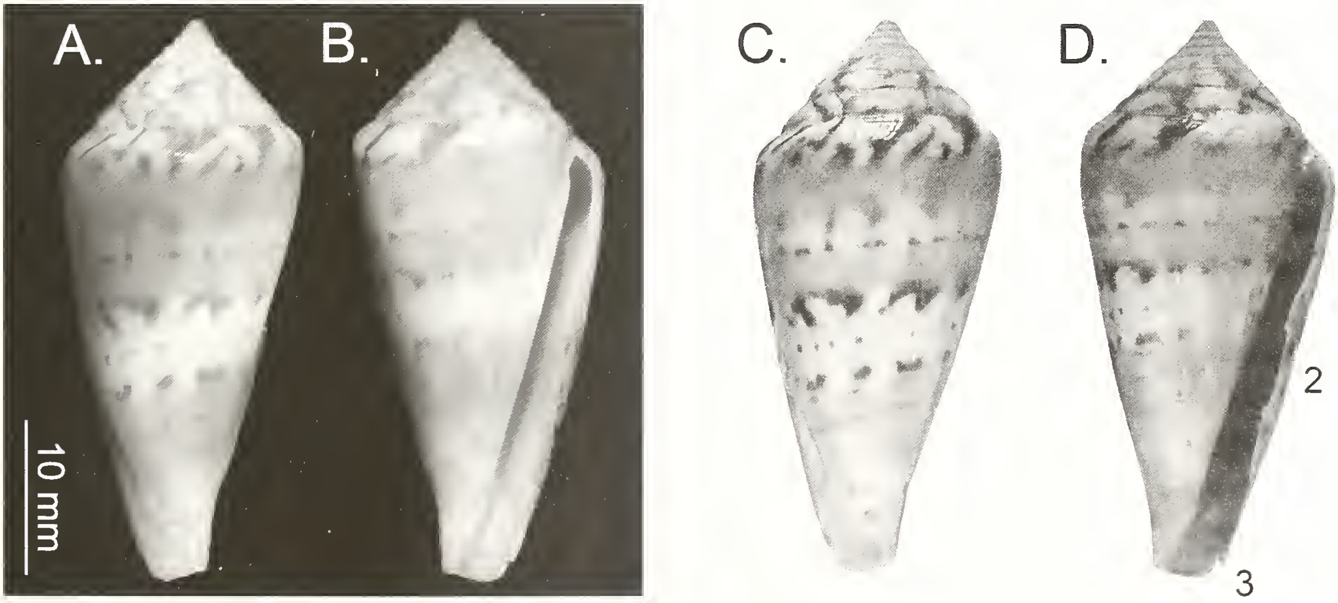

Figure 1. The holotype of *Conus lightbourni* Petuch, 1986. Dorsal (A) and ventral (B) view of the returned specimen. The shell markings are an obvious match to the dorsal (C) and ventral (D) view of Petuch (1986: fig. 1). Three areas of damage to the outer lip are identified by number for comparison with Fig. 2. Original © 2008 Biological Society of Washington, from the *Proceedings of the Biological Society of Washington*. Reprinted by permission of Allen Press Publishing Services.

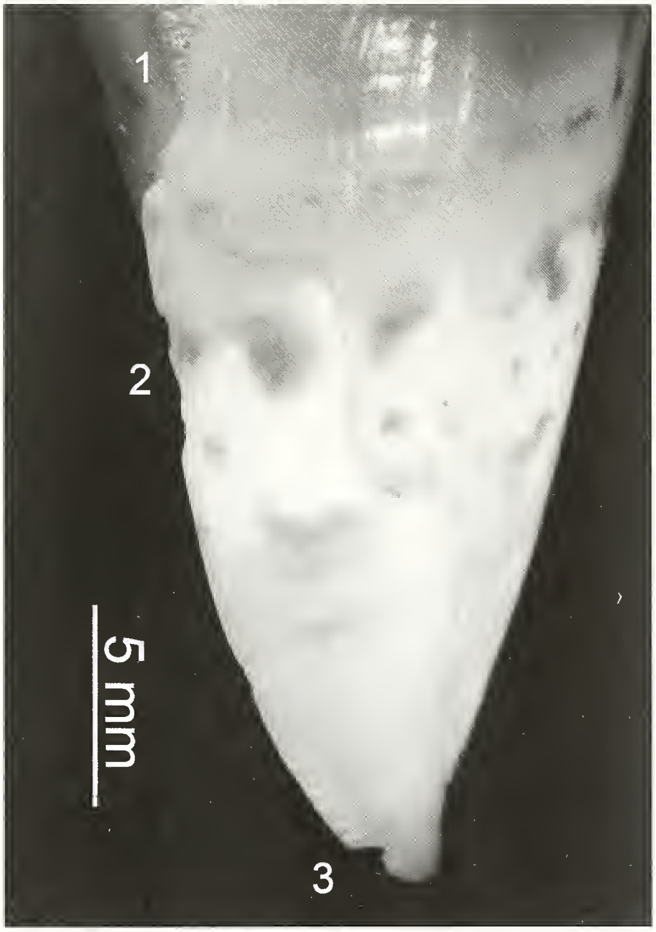

Figure 2. The holotype of *Conus lightbourni*, lateral view. As in the original photograph, the outer lip is damaged at three locations. Ongoing wear at the outer lip makes the damage at location 2 appear diminished.

wide salmon-pink bands, and brown flammules and dots. Three paratypes (26.0-47.7 mm length) were designated as coming from the same depth and locality as the holotype, and five additional specimens (22.4-44 mm) were examined but not included as paratypes.

In 2007, Don Pisor purchased Jack Lightbourn's private collection, with one known specimen of *Conus lightbourni* as part of the transaction. Upon unpacking, a second shell was found in a box of miscellaneous Bermuda specimens and other items (Pisor, pers. comm.). One of these two specimens was sold to Bill Fenzan who recognized it as the holotype. After an independent confirmation of the identification, the holotype (Figs. 1-2) was returned to DMNH on 14 March 2008.

The color and breakage patterns of the returned specimen are a convincing match to the original holotype description (Figs. 1-2). The shell length and maximum diameter (Kohn and Riggs 1975) of the returned shell were measured using NIS-Elements D 3.00 digital imagery software. The length, measured from the top of the spire to the base of the columella, is 35.9 mm, within 2.5% of the original measurement. The maximum diameter is 16.2 mm, within 1.2% of the original measurement. Damage at the edge of the outer lip is consistent in location, but different in degree (Figs. 1-2). The returned specimen has brown flammules and dots, which match the original description, but the salmon-pink bands are orange-brown and the base color appears white. These differences are consistent with wear from improper long-term storage and fading due to exposure to light and do not change the conclusion that the returned specimen is the holotype of *Conus lightbourni*.

ACKNOWLEDGMENTS

We thank Mr. Don Pisor for facilitating the return of the holotype to DMNH and Dr. Harry Lee for his independent evaluation of the identification. Reviews of this research note by Dr. Rüdiger Bieler and Dr. Tom Duda and discussions with Dr. Alan Kohn are greatly appreciated.

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INDEX TO VOLUME 26

AUTHOR INDEX

Averbuj, A. 26: 67
Beugly, J. 26: 137
Chapman, E. J. 26: 161
Chenoweth, M. S. 26: 171
Dourson, D. C. 26: 153
Fenzan, W. J. 26: 179
Galván-Villa, C. M. 26: 119
Gray, S. M. 26: 19
Hayes, D. M. 26: 171
Hill, A. M. 26: 171
Johnsen, S. 26: 27
Kelly, S. 26: 19
López-Uriarte, E. 26: 119
Martin, E. 26: 137
Meyer-Rochow, V. B. 26: 47
Minton, R. L. 26: 171
Morton, B. 26: 35
Pearce, T. A. 26: 111
Penchaszadeh, P. E. 26: 67
Piatigorsky, J. 26: 73
Pyron, M. 26: 137
Ríos-Jara, E. 26: 119
Robles, L. J. 26: 19
Serb, J. M. 26: 1, 3
Shea, E. K. 26: 179
Smith, T. A. 26: 161
Speiser, D. I. 26: 27
Spielman, M. 26: 137
Voight, J. R. 26: 133
Salvini-Plawen, L. V. 26: 83
White, J. D. 26: 171
Wilkens, L. A. 26: 101
Zieger, M. V. 26: 47

PRIMARY MOLLUSCAN TAXA INDEX

[first occurrence in each paper recorded, new taxa in bold]

acapulcensis, Nuculana 26: 123
achatina, Achatina 26: 49
acicula, Cecilioides 26: 48
acuta, Physella 26: 145
acuta, Pleurocera 26: 142
adamsi, Anadara 26: 119
aenigmatica, Enigmonia 26: 38
aequatorialis, Anadara 26: 124
Aeropneusta 26: 83
agreste, Deroceras 26: 49
albolabris, Neohelix 26: 112
aletes, Pitar 26: 124
alternata, Anguispira 26: 112
americanus, Spondylus 26: 1, 27, 101
Amphitretus 26: 10
Amusium 26: 39
Anadara 26: 93, 125
Anatina 26: 94
anceps, Helisoma 26: 146, 173
ancillaria, Physella 26: 149
Ancylidae 26: 148, 173
annulatum, Lucinoma 26: 130
Anomalodesmata 26: 35, 94
Anomiidae 26: 38
Anomioidea 26: 35, 93
antrosum, Helisoma 26: 149
aphanes, Neophysema 26: 130
Aplysia 26: 11, 56, 86
approximata, Parvilucina 26: 119
Arca 26: 93
Architaenioglossa 26: 173
Arcidae 26: 6, 37, 89, 121
Arcoida 26: 7, 41, 91, 106
Arcoidea 26: 35, 93
arcuata, Pandora 26: 131
arenaria, Mya 26: 39, 96, 102
armigera, Planorbula 26: 148
aspersa, Helix 26: 116
aspersum, Cornu 26: 47
atra, Aulacomya 26: 35
aurora, Psammotreta 26: 124
balloti, Amusium 26: 1, 27, 101
Barbatia 26: 93
Basommatophora 26: 11, 173
Bathothauma 26: 10, 133
beebei, Cyclocardia 26: 130
belcheri, Trachycardium 26: 124
berryi, Pitar 26: 124
bicarinata, Valvata 26: 139
bimaculoides, Octopus 26: 1, 20
biolleyi, Leptopecten 26: 129
bitentaculatus, Athoracophorus 26: 53
Bivalvia 26: 1, 7, 35, 83
brevialata, Pteria 26: 37
brevifrons, Lunarca 26: 124
Buccinanops 26: 2, 67
Buccinum 26: 67
bulimoides, Fossaria 26: 173
Bullia 26: 67
Caenogastropoda 26: 2, 67, 91
californica, Aplysia 26: 11, 52, 92, 103
californica, Gouldia 26: 119
californicus, Ariolimax 26: 53
callicomatus, Pitar 26: 131
campanulata, Planorbella 26: 147
campanulatum, Helisoma 26: 149
campbellorum, Semelina 26: 123
Campeloma 26: 139
canaliculata, Pleurocera 26: 173
cancellaria, Barbatia 26: 6, 41
cancellaris, Radiolucina 26: 119
caperata, Lymnaea 26: 149
caperata, Stagnicola 26: 144
Cardiidae 26: 39, 94, 130
Cardiinae 26: 94
Cardioidea 26: 91
Carditidae 26: 130
cardium, Lampsilis 26: 166
Carinaria 26: 86
carpenteri, Tellina 26: 131
catascopium, Lymnaea 26: 149
catascopium, Stagnicola 26: 143
catskillensis, Discus 26: 112
Caudofoveata 26: 89

Cecilioides 26: 67
centrifuga, Lucinisca 26: 123
Cephalopoda 26: 7, 84, 133
Chamidae 26: 125
chinensis, Bellamya 26: 139
Chione 26: 125
Chitonidae 26: 7
Chlamys 26: 36
cicercula, Strigilla 26: 124
cincinnatiensis, Pomatiopsis 26: 141
circumstriatus, Gyraulus 26: 145
clava, Pleurobema 26: 161
Clavagellidae 26: 35
coani, Tellina 26: 131
cochlidium, Buccinanops 26: 67
Coleoida 26: 92
columella, Lymnaea 26: 149
columella, Pseudosuccinea 26: 144, 173
compta, Chione 26: 123
concavum, Haplotrema 26: 112
Conchifera 26: 89
concinna, Anadara 26: 129
concinnus, Pitar 26: 124
contectoides, Valvata 26: 149
Corbulidae 26: 131
corneus, Planorbarius 26: 48
Cornu 26: 47
costata, Lasmigona 26: 166
Cranchiidae 26: 133
Crassatellidae 26: 129
Ctenoides 26: 93
Cucullaea 26: 92
Cucullaeidae 26: 92
cupreus, Mesomphix 26: 153
Cylichna 26: 67
dalli, Lymnaea 26: 149
Decabrachia 26: 92
decisum, Campeloma 26: 139, 173
declivis, Nucula 26: 123
decussata, Crenella 26: 129
deflectus, Gyraulus 26: 146
delgada, Sheldonella 26: 129
denotatum, Xolotrema 26: 153
dentifera, Neohelix 26: 155
dichotoma, Strigilla 26: 124
dilatata, Elliptio 26: 164
dilatatus, Micromenetus 26: 173
discus, Haliotis 26: 91
distinctus, Arion 26: 57
divaricata, Lacuna 26: 91
Docoglossa 26: 88
Donacidae 26: 131
dunkeri, Dosinia 26: 124
ecuadoriana, Crassinella 26: 124
edentuloides, Pegophysema 26: 130
edule, Cardium 26: 101
edule, Cerastoderma 26: 39, 95
edulis, Mytilus 26: 89
edulis, Ostrea 26: 36
elenense, Laevicardium 26: 124
Ellobiacea 26: 48
elodes, Stagnicola 26: 144
elongata, Aplexa 26: 145
elytrum, Macoma 26: 131
Enigmonia 26: 93
Eupulmonata 26: 92
Euthyneura 26: 91
exacuous, Menetus 26: 149
exacuous, Promenetus 26: 148
excavata, Conchocele 26: 124
exilis, Lymnaea 26: 49
exilis, Stagnicola 26: 144
fallax, Triodopsis 26: 112
fascicularis, Acanthochiton 26: 91
fasciola, Lampsilis 26: 166
fasciolaris, Ptychobranchus 26: 164
fenestrata, Lucinisca 26: 124
Ferussaciidae 26: 67
Fissurella 26: 89
flavus, Limax 26: 49
flexuosa, Thyasira 26: 130
floridanus, Ctenoides 26: 38
fluviatilis, Ancylus 26: 48
fontinalis, Physa 26: 48
formosa, Anadara 26: 129
Fossaria 26: 143
fragilis, Ferrissia 26: 139
fraternum, Euchemotrema 26: 112
fulgens, Ovachlamys 26: 111
fulica, Achatina 26: 49
fuscus, Laevapex 26: 148, 173
Gastropoda 26: 2, 7, 62, 67, 86
georgianus, Viviparus 26: 139
gigantea, Crassodoma 26: 27, 101
gigantea, Lottia 26: 11
gigas, Crassostrea 26: 36
glabrata, Biomphalaria 26: 11
Glycimeridae 26: 92
Glycimeris 26: 92
Glycymerididae 26: 129
gouldiana, Bulla 26: 57
gracilis, Donax 26: 124
gradata, Acar 26: 129
granifera, Trigoniocardia 26: 124
guatulcoensis, Chione 26: 130
Gymnomorpha 26: 92
gyrina, Physella 26: 145, 173
Haliotis 26: 67, 88
hastata, Chlamys 26: 27
Heteropoda 26: 86
heterostropha, Physella 26: 149
hirsutus, Gyraulus 26: 149
hispida, Trichia 26: 48
Histioteuthis 26: 10
humilis, Lymnaea 26: 149
hyalina, Lyonsia 26: 95
Hydrobiidae 26: 140, 173
hypnorum, Aplexa 26: 149
Ilyanassa 26: 88
inermis, Navanax 26: 49
integra, Cincinnatia 26: 140, 173
integra, Physella 26: 149
interrupta, Strigilla 26: 131
intertextus, Viviparus 26: 173
ira, Corbula 26: 119
iris, Villosa 26: 161
irradians, Argopecten 26: 6, 27, 101
Isognomon 26: 93
Isognomonidae 26: 129
jaeckeli, Albinaria 26: 111
japonica, Bellamya 26: 139
kellettii, Lirophora 26: 124
Kellidae 26: 130
kusceri, Congeria 26: 36
lactea, Otala 26: 60
lactea, Striarca 26: 92
laeviradius, Nuculana 26: 119
Laternula 26: 94
Laternulidae 26: 1, 35
laticostata, Cardites 26: 130
Leachia 26: 135
Leptopecten 26: 39
lewisi, Valvata 26: 139
lewisii, Valvata 26: 149
lightbourni, Conus 26: 179
lightbourni, Floraconus 26: 179
Lima 26: 83, 101
lima, Lima 26: 83
Limacidae 26: 9
Limidae 26: 8

Limoidea 26: 35, 91
Limopsidae 26: 92
Limopsoidea 26: 35
limosa, Amnicola 26: 149
limosus, Amnicola 26: 149
Littorina 26: 6
livescens, Elimia 26: 141
livescens, Goniobasis 26: 149
lobula, Nuculana 26: 119
Loligo 26: 10
Lucinidae 26: 121
lucorum, Helix 26: 57
lustrica, Amnicola 26: 140
lustrica, Pyrgulopsis 26: 149
Lymnaeidae 26: 143, 173
Lyonsiidae 26: 95
lyromma, Bathothauma 26: 133
macclintocki, Discus 26: 158
Mactridae 26: 131
magellenicus, Placopecten 26: 27
marginata, Alasmidonta 26: 161
mariae, Lirophora 26: 124
marmorata, Corbula 26: 124
martinicensis, Tellina 26: 131
maxima, Tridacna 26: 39, 101
maximus, Limax 26: 49
maximus, Pecten 26: 27, 39, 93, 101
mazatlanica, Parvilucina 26: 119
meekiana, Gundlachia 26: 149
megastropha, Strophocardia 26: 124
mercenaria, Mercenaria 26: 101
Minnivola 26: 39
modesta, Transennella 26:
multicostata, Periglypta 26:
multicostata, Tucetona 26:
multispinosus, Pitar 26:
munita, Philobrya 26:
Murex 26: 88
murrayi, Cirrothauma 26: 10
mutica, Pterotrachea 26: 86
Myidae 26: 39, 91
Myoida 26: 96
mystica, Cryptoplax 26: 91
Mytilidae 26: 36, 119
Mytiloidea 26: 35
nasuta, Corbula 26: 119
Nautilus 26: 9, 92
neglectus, Onithochiton 26: 7
nemoralis, Cepaea 26: 48, 158
Neogastropoda 26: 2
Neotaenioglossa 26: 173
Neritilidae 26: 91
Neritiliidae 26: 67
neritoides, Latia 26: 48
Neritopsina 26: 91
noae, Arca 26: 6, 37
Noetiidae 26: 129
notabilis, Anadara 26: 6, 42
novemcostatus, Trigonulina 26: 131
Nuculana 26: 125
Nuculanidae 26: 123
Nuculidae 26: 129
nux, Anadara 26: 124
obesa, Anadara 26: 124
oblongum, Cardium 26: 39
obovalis, Trigoniocardia 26: 130
obovata, Lithasia 26: 137
obsoleta, Ilyanassa 26: 86
Octobrachia 26: 92
Octopus 26: 88
Oegopsida 26: 133
officinalis, Sepia 26: 10
Olivancillaria 26: 69
Olividae 26: 69
olssoni, Sheldonella 26: 124
Onchidiidae 26: 92
Onchidium 26: 92
Opisthobranchia 26: 11
orcutti, Fartulum 26: 91
Ostrea 26: 36
Ostreidae 26: 89
Ostreoidea 26: 35
ovalis, Novisuccinea 26: 112
pacifica, Arca 26: 125
pacifica, Crassinella 26: 119
pacifica, Leachia 26: 135
pacifica, Tellina 26: 124
pallida, Mulinia 26: 124
pallida, Semele 26: 131
palustris, Lymnaea 26: 149
Pandoridae 26: 94, 131
Pandoroidea 26: 42, 91
parallela, Ferrissia 26: 149
parallelus, Ferrissia 26: 149
parva, Amnicola 26: 149
parva, Lymnaea 26: 149
parvus, Gyraulus 26: 146
Patella 26: 88
Patellidae 26: 91
Patellogastropoda 26: 11
Pecten 26: 13
Pectinidae 26: 1, 7, 35, 93, 101, 125
Pectinoidea 26: 27, 35, 91
pectunculoides, Bathyarca 26: 41
Pectunculus 26: 92
pencillata, Plicatula 26: 124
Penicillidae 26: 35
peregra, Radix 26: 48
pernomus, Cyclopecten 26: 119
peroni, Atlanta 26: 86
perparvula, Divalinga 26: 130
peslutre, Pseudamussium 26: 83
pespelicani, Aporrhais 26: 92
pharaonis, Sepia 26: 11
Philobrya 26: 37, 92
Physidae 26: 145, 173
pisana, Euparipha 26: 60
pisana, Helix 26: 60
Pisulina 26: 67
Placopecten 26: 27, 39
Placophora 26: 2, 7, 87
Planorbidae 26: 145, 173
platysayoides, Triodopsis 26: 153
Pleurobranchus 26: 88
Pleuroceridae 26: 141, 173
pleuronectes, Amusium 26: 39
Plicatulidae 26: 125
politus, Tagelus 26: 124
Polyplacophora 26: 2, 7, 96
pomatia, Helix 26: 55
Pomatiopsidae 26: 141
ponderosa, Dosinia 26: 130
praerosa, Anculosa 26: 149
praerosa, Leptoxis 26: 143
pristiphora, Tellina 26: 124
procerum, Trachycardium 26: 124
prolongata, Lucina 26: 124
Propeammusiidae 26: 129
Protobranchia 26: 89
Pseudamussium 26: 83
pseudoflavus, Limax 26: 57
Pteriidae 26: 37, 129
Pterioida 26: 106
Pterioidea 26: 35
Pteriomorpha 26: 89
Pteriomorphia 26: 39
pulicaria, Chione 26: 124
pulicaria, Chionopsis 26: 130
punctata, Aplysia 26: 92
pusio, Chlamys 26: 93

pusio, Pecten 26: 38
putris, Succinea 26: 53
radiata, Lampsilis 26: 166
recognitus, Isognomon 26: 124
reeveana, Barbatia 26: 124
reflexa, Lymnaea 26: 149
reticulatum, Deroceras 26: 57
reticulatus, Agriolimax 26: 49
reticulatus, Nassarius 26:
Retusa 26: 67
Retusidae 26: 67
rivularis, Ferrissia 26: 173
rubida, Chlamys 26: 27, 101
rufus, Arion 26: 52
sayii, Physella 26: 149
scabra, Lima 26: 38, 93, 101
scalariformis, Cerithidea 26: 92
Scaphandridae 26: 67
Scaphopoda 26: 89
schenki, Nucula 26: 124
scolopes, Euprymna 26: 11, 75
Semelidae 26: 131
semicarinata, Elimia 26: 141
senticosum, Trachycardium 26: 124
siliqua, Macoma 26: 124
sincera, Valvata 26: 139
singleyanus, Helicodiscus 26: 48
sintoxia, Pleurobema 26: 161
Smaragdia 26: 88
Solecurtidae 26: 131
Solenogastres 26: 89
solidissima, Spisula 26: 11, 35, 101
sordida, Chama 26: 125
soror, Diplodonta 26: 130
Spondylidae 26: 8, 42, 93
Spondylus 26: 39
squalida, Megapitaria 26: 123
squamosa, Lima 26: 93
stagnalis, Lymnaea 26: 48, 143
sterna, Pteria 26: 125
striata, Dyakia 26: 60
striata, Quantula 26: 60
strigilata, Tucetona 26: 129
Strigilla 26: 124
Strombus 26: 13, 88
Strophochelius 26: 52
subalata, Mactrellona 26: 124
subglobosus, Birgella 26: 140
subglobosus, Somatogyrus 26: 149
suborbicularis, Kellia 26: 124
subpurpureus, Valvata 26: 139
subpurpureus, Viviparus 26: 173
subquadrata, Cyclinella 26: 130
subquadrata, Diplodonta 26: 130
subrotunda, Obovaria 26: 161
tantilla, Nutricola 26: 41
Taoniinae 26: 133
tarda, Ferrisia 26: 149
Tellinidae 26: 121
Testaria 26: 90
Thyasiridae 26: 130
thyroidus, Mesodon 26: 112
tricarinata, Valvata 26: 139
Tridacna 26: 93, 101
Tridacnidae 26: 39
Tridacninae 26: 94
Tridacnoidea 26: 35
tridentata, Triodopsis 26: 155
triquetra, Epioblasma 26: 161
trivolvis, Helisoma 26: 149
trivolvis, Planorbella 26: 147, 173
Trochacea 26: 67
truncata, Laternula 26: 40
Tryblidia 26: 89
undulata, Cymatoica 26: 130
undulatus, Strophitus 26: 165
Ungulinidae 26: 130
Valvatidae 26: 139
varia, Chlamys 26: 83
varians, Crassinella 26: 124
velero, Leptopecten 26: 129
Veneridae 26: 121
Veneroida 26: 11, 95
ventricosa, Corbula 26: 131
ventricosus, Argopecten 26: 125
Venus 26: 102
verrucosa, Semele 26: 124
verruculatum, Onchidium 26: 51, 93, 103
Verticordiidae 26: 131
Vetigastropoda 26: 88
vincta, Lacuna 26: 91
virescens, Barbatia 26: 37
virginica, Crassostrea 26: 36
Visceroconcha 26: 92
vittatus, Donax 26: 36
Viviparidae 26: 139, 173
viviparous, Viviparus 26: 91
vulgaris, Octopus 26: 11, 43
walkeri, Physella 26: 149
yessoensis, Patinopecten 26: 93
zebra, Arca 26: 6
ziczac, Euvola 26: 107
ziczac, Pecten 26: 107

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Hillis, D. M. 1989. Genetic consequences of partial self fertilization on populations of *Liguus fasciatus* (Mollusca: Pulmonata: Bulimulidae). *American Malacological Bulletin* **7**: 7-12.

Seed, R. 1980. Shell growth and form in the Bivalvia. *In*: D. C. Rhoads and R. A. Lutz, eds., *Skeletal Growth of Aquatic Organisms*. Plenum Press, New York, New York. Pp. 23-67.

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July 19 - 23 2009

The 75th meeting of the American Malacological Society will be held in Ithaca, New York from July 19th through July 23rd, 2009. The meeting will be held at Cornell University.

Special events will be held all around Ithaca, including an opening reception at the Museum of the Earth at the Paleontological Research Institution, a dinner cruise on scenic Cayuga Lake, the AMS auction on Tuesday at the Holiday Inn in Downtown Ithaca, and a banquet at the Museum of the Earth on Wednesday evening.

The large recent and fossil mollusk collections of PRI will be open throughout the week for scientific work. Please visit the Collections website at www.pricollectionsdatabase.org.

The American Malacological Society

75th Annual Meeting
July 19th - July 23rd, 2009
Ithaca, New York

This meeting's field trips will familiarize meeting participants with Ithaca's aquatic habitats, both ancient and modern. Chose between exploring the Devonian marine fossils of the Ithaca Region with Warren Allmon and the freshwater and land mollusks of the Cayuga Lake Basin with Eileen Jokinen.

Participants flying to Ithaca can chose among the Ithaca, Binghamton, Syracuse, or Rochester airports. Binghamton and Syracuse are both approximately an hour by car from Ithaca, and Rochester is two hours away. Housing is available either at the Holiday Inn Downtown, or on campus in residence halls at Cornell University. Transportation will be provided to all events from each location.

For registration and abstract submission information, please visit www.malacological.org
We look forward to seeing you in Ithaca in 2009!

For more information, please contact:
Kelly Cronin
PRI
1259 Trumansburg Rd
Ithaca, Ny 14850
E-mail: cronin@museumoftheearth.org
Phone: 607-273-6623 ext. 10

The American Malacological Society

75th Annual Meeting
July 19th - July 23rd, 2009
Ithaca, New York

Symposium on Speciation in Mollusks

This multi-disciplinary symposium aims to bring together researchers working on molluscan speciation in a variety of taxa, at a variety of temporal and geographic scales, and with a variety of techniques — from fossils to genes.

Confirmed speakers include Warren Allmon (Paleontological Research Institution, Ithaca, NY), Robert Dillon (College of Charleston, Charleston, SC), Matthias Glaubrecht (Humboldt University, Berlin), Patrick Krug (California State University, Los Angeles), Peter Marko (Clemson University, Clemson, SC), Elinor Michel (The Natural History Museum, London), Paula Mikkelsen (Paleontological Research Institution, Ithaca, NY), Rebecca Rundell (University of British Columbia, Vancouver), and Ursula Smith (Cornell University, Ithaca, NY).

Please visit the meeting website at www.malacological.org for more information on symposium topics, accommodations, and things to do in and around Ithaca.

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Photoreception and the polyphyletic evolution of photoreceptors (with special reference to Mollusca). **LUITFRIED VON SALVINI-PLAWEN** 83

Primary inhibition by light: A unique property of bivalve photoreceptors. **LON A. WILKENS** 101

When a snail dies in the forest, how long will the shell persist? Effect of dissolution and micro-bioerosion. **TIMOTHY A. PEARCE** 111

Bivalve molluscs from the continental shelf of Jalisco and Colima, Mexican Central Pacific. **EDUARDO RÍOS-JARA, ERNESTO LÓPEZ-URIARTE, and CRISTIAN M. GALVÁN-VILLA** 119

A mature female of *Bathothauma* Chun, 1906 (Cephalopoda: Cranchiidae) from Hawaii. **JANET R. VOIGHT** 133

Conservation of the freshwater gastropods of Indiana: Historic and current distributions. **MARK PYRON, JAYSON BEUGLY, ERIKA MARTIN, and MATTHEW SPIELMAN** 137

The feeding behavior and diet of an endemic West Virginia land snail, *Triodopsis platysayoides*. **DANIEL C. DOURSON** 153

Structural community changes in freshwater mussel populations of Little Mahoning Creek, Pennsylvania. **ERIC J. CHAPMAN and TAMARA A. SMITH** 161

Diversity and distribution of freshwater gastropods in the Bayou Bartholomew drainage, Arkansas, U.S.A. **RUSSELL L. MINTON, JOHN D. WHITE, DAVID M. HAYES, M. SEAN CHENOWETH, and ANNA M. HILL** 171

Research Note: *Conus lightbourni* holotype returned to the Delaware Museum of Natural History. **ELIZABETH K. SHEA and WILLIAM J. FENZAN** 179

Index to Vol. 26 181

Membership Form for 2009 185

Information for Contributors 187

Meeting Announcement 189

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